Study on Mechanism Why Rats are Hypo-responsive but Hamsters are Hyper-responsive to Dietary Cholesterol

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DECLARATION

The experiments reported in this dissertation were carried out in the Department of Biochemistry, the Chinese University of Hong Kong, between August 2003 and July 2005. This work is solely that of the author. No part of this dissertation is being concurrently submitted for any other degree, diploma or other qualification at this or any other institutions.

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ABBREVIATIONS

AMP-LB	ampicillin added luria broth
ANOVA	one-way analysis of variance
APO	apolipoprotein
bHLH-ZIP	basic helix-loop-helix leucine zipper
cDNA	complementary deoxyribonucleic acid
CHD	coronary heart disease
CYP7A1	cytochrome P450 7A1; cholesterol-7a-hydroxylase
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
DEPC	diethyl pyrocarbonate
dGTP	2'-deoxyguanosine 5'-triphosphate
DIG	digoxigenin
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphates = nucleotides
DTT	dithiothreitol
dTTP	2'-deoxythymidine 5'-triphosphate
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
EST	expressed sequence-tagged
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GLC	gas-liquid chromatograph
GS	golden syrian
HMG-COA	3-hydroxy-3-methylglutaryl-CoA

IgG	immunoglobulin g
IPTG	isopropyl β-D-1-thiogalactopyranoside
LB	luria broth
LDL	low density lipoprotein
LDL-C	low density lipoprotein cholesterol
LDL-receptor	low density lipoprotein receptor
LXR	liver x receptor
LXRE	liver X receptor responsive element
LXR-α	liver x receptor-alpha
mRNA	messenger ribonucleic acid
NBT/BCIP	4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate
NF-1	nuclear factor 1
nSREBP-2	mature form of sterol regulatory element binding protein-2
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pSREBP-2	precursor form of sterol regulatory element binding protein-2
r^2	coefficient of determinant = r squared
RNA	ribonucleic acid
RXRs	retinoid X receptors
S.E.M.	standard error of means
S1P	site-1 protease
S2P	site-2 protease
SD	sprague dawley
SDS	sodium dodecyl sulfate
SRE	sterol regulatory element

SREBP-2	sterol regulatory element binding protein-2
SSC	sodium chloride citrate
TAE	tris-acetate/edta electrophoresis buffer
TBS	tris-buffered saline
TC	total cholesterol
TMS	trimethyl-sily
TRIS	tris(hydroxymethyl)aminomethane
UV	ultraviolet
X-GAL	5-bromo-4-chloro-3-indoyl-beta-d-galactopyranoside

ABSTRACT

Coronary Heart Disease (CHD) is the most important clinical manifestation of atherosclerosis. The most prominent risk factor for development of atherosclerosis is hypercholesterolemia. Lower serum cholesterol is believed to have lower risk of CHD. The present thesis project studied effects of chemical cholesterol incorporated into high cholesterol diets on serum cholesterol level in rats (Sprague-Dawley rat) and hamsters (Golden Syrian hamsters, *Mesocricetus auratus*). It was found that the rats were hypo-responsive to dietary cholesterol but hamsters were hyper-responsive.

The first part of study investigated the molecular mechanism by which rats and hamsters had different response toward dietary cholesterol. The present study examined the following proteins/mRNAs, which involved in cholesterol metabolism: (a) nuclear form of sterol regulatory element-binding protein-2 (nSREBP-2), which regulates gene expression of protein/enzymes involved in cholesterol homeostasis; (b) LDL-receptor, which mediates the removal of circulating cholesterol; and (c) HMG-CoA reductase, which is a key enzyme in cholesterol synthesis; (d) CYP7A1, which is a regulatory enzyme in conversion of cholesterol to bile acids; and (e) LXR-α, which is the positive regulator of CYP7A1. Western blot showed that nSREBP-2, LDL-receptor, and CYP7A1 expression were enhanced in rats but not in hamsters when dietary cholesterol increased. Northern blot further confirmed that the up-regulation of LDL-receptor and CYP7A1 occurred at transcription level in rats, by nSREBP-2 and LXR-α respectively. Taken together, the present results demonstrated that rats were hypo-responsive to dietary cholesterol because excess cholesterol in blood was transported by LDL-receptor into the liver and excess cholesterol in the liver was excreted as bile acids via CYP7A1 pathway. In contrast, hamsters were hyper-responsive because they did not show corresponding change in LDL-receptor

and CYP7A1 when exposed to a high dietary cholesterol diet and the cholesterol accumulated in blood.

Most interesting observation was that different responsiveness occurred in the same species. The second part of the study was performed to determine the mechanism for individual variation of serum cholesterol in response to a same amount of dietary cholesterol. The correlation between protein expression of LDL-receptor, CYP7A1, nSREBP-2, LXR-α, HMG-CoA reductase and serum total cholesterol was investigated in both rats and hamsters. It was found that higher CYP7A1 protein expression was linked with lower serum total cholesterol in both rats and hamsters, suggesting efficient cholesterol elimination was common for those hypo-responders. LDL-receptor protein expression was negative correlated with serum total cholesterol in hamsters, implying that high LDL-receptor protein expression was an additional mechanism for lowering serum total cholesterol in hamsters. However, no correlation of nSREBP-2, LXR-α, HMG-CoA reductase with serum total cholesterol was observed in rats or hamsters, indicating that serum total cholesterol level was not directly influenced by the expression of these proteins.

To summarize, both the efficient elimination of cholesterol from the body (via CYP7A1 pathway) and the efficient removal of serum cholesterol by LDL-receptor are important characteristics for the hypo-responsiveness of individuals to dietary cholesterol.

摘要

冠心病(Coronary Heart Disease, CHD)是動脈粥樣硬化(Atherosclerosis)最重要的臨床表現，而高膽固醇症(hypercholesterolemia) 是引起動脈粥樣硬化最主要的原因。所以，降低血清膽固醇可助於降低冠心病的風險。本研究比較了大鼠(Sprague-Dawley rat) 與倉鼠(Golden Syrian hamster) 的血清膽總固醇含量對高膽固醇膳食的反應。研究發現大鼠對膳食膽固醇屬於低反應性(hypo-responsive)，而倉鼠則爲高反應性(hyper-responsive)。

研究的第一部份，是探討大鼠及倉鼠對膳食膽固醇不同反應的分子機理(molecular mechanism)。我們研究了下列涉及膽固醇代謝的蛋白質和信使核糖核酸(mRNA)：(一) 核型膽固醇調節元件结合蛋白-2 (nSREBP-2)，它能調節涉及體內膽固醇穩態的蛋白質及酶；(二) 低密度脂蛋白受體 (LDL-receptor) ，它能控制將血液循環中的膽固醇轉運至肝臟；(三) HMG-CoA 還原酶 (HMG-CoA reductase) ，它是膽固醇生物合成中的一種關鍵酶；(四) 膽固醇-7α-羥基化酶 (CYP7A1) ，是一種膽汁酸鹽生物合成的限速酶；(五) 肝 X 受體 (Liver x receptor)，它能正相調節膽固醇-7α-羥基化酶。Western blot 的結果顯示大鼠的膽固醇調節元件结合蛋白-2、低密度脂蛋白受體和膽固醇-7α-羥基化酶隨膳食中的膽固醇水平升高而增加，但倉鼠卻沒有增加。Northern blot 的結果進一步證明低密度脂蛋白受體和膽固醇-7α-羥基化酶的增加是在轉錄水平的，並分別受核型膽固醇調節元件结合蛋白-2 及肝 X 受體調節。總之，我們的數據顯示大鼠對膳食內膽固醇的低反應性是由於過多的膽固醇被低密度脂蛋白受體從血液循環轉運至肝臟，進而被膽固醇-7α-羥基化酶轉化成膽汁酸鹽排出體外。相反，倉鼠的高反應性是由於低密度脂蛋白受體和膽固醇-7α-羥基化酶對膳食內的膽固醇無反應，膳食中的膽固醇在循環系統蓄積而引起的。

最令人關注的是同一種屬的反應性也不相同。實驗的第二部分是研究膳食中

的等量膽固醇對血清總膽固醇的影響，同時也研究了涉及膽固醇代謝的多種蛋白質表達與血清總膽固醇在大鼠和倉鼠模型中的關係。結果表明膽固醇-7α-羥基化酶的高表達與大鼠和倉鼠的低血清總膽固醇有相關，提示有效的膽固醇排出是低反應者的共同特點。低密度脂蛋白受體的表達水平與血清總膽固醇在倉鼠呈負相關，提示低密度脂蛋白受體的表達是低反應性的另一機理。然而，核型膽固醇調節元件结合蛋白-2、HMG-CoA 還原酶和肝 X 受體蛋白表達對大鼠和倉鼠的血清總膽固醇都沒有關聯。該結果表明血清總膽固醇水平並不直接受這些蛋白質表達的影響。

總結而言，經膽固醇-7α-羥基化酶途徑從身體有效地排出膽固醇和經低密度脂蛋白受體把血液循環中的膽固醇有效地轉運至肝臟是對膳食膽固醇低反應性的共同點。

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CHAPTER 1

General Introduction

1.1 Cholesterol

1.1.1 History of cholesterol

The cholesterol was first recognized as an alcohol-soluble, readily crystallizable component of bile stones by Chevreul in 1816. It was first named "cholesterine" from two Greek words: chole, meaning bile, and stereos, meaning solid. In the middle of nineteenth century, cholesterin was also identified as a constituent of several normal animal tissues and in atheromatous lesions of arteries. The name was changed to "cholesterol" in English-speaking countries in the twentieth century.

1.1.2 Structure of cholesterol

The structure of cholesterol was elucidated by the researches of Wieland and Windaus extending over a quarter of a century and finally established by crystallography (Bernal, 1932). Cholesterol is a steroid that built from four linked hydrocarbon rings. A hydrocarbon tail is linked to the steroid at one end, and a hydroxyl group is attached at the other end. Two angular methyl groups, C-18 and C-19, attached to C-13 and C-10 of the hydrocarbon ring, respectively. The C-18 and C-19 methyl groups lie above the plane containing the four rings. Figure 1.1 showed the chemical structure of cholesterol.

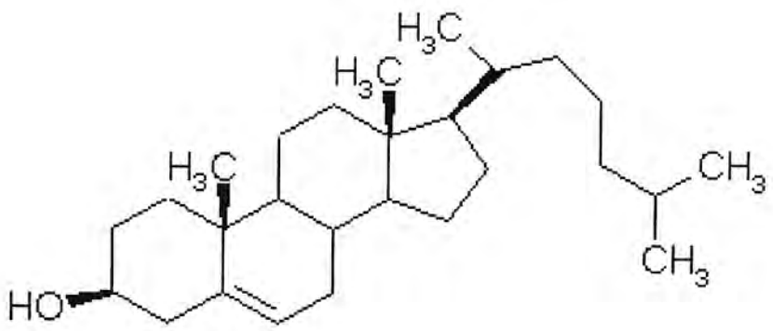

Figure 1.1. Chemical structure of cholesterol

1.1.3 Biological function of cholesterol

Cholesterol serves as important component of the cell membrane, precursor of steroid hormones and bile acids. As a necessary component of biological membranes, cholesterol provides stability that makes the membrane's fluidity stable over a bigger temperature interval. This was done by breaking up the *Van der Waals* interactions and the close packing of the phospholipid tails, which makes the membrane more fluid. Hence, one way for a cell to control the fluidity of its membrane is by regulating its level of cholesterol in the cell membrane.

Cholesterol is the major precursor for many steroid hormones. Five major classes of steroid hormones are derived from cholesterol: progestagens, glucocorticoids, mineralocorticoids, androgens, and estrogens. In the adrenal glands, cholesterol is converted to cortisol and aldosterone, which controls the carbohydrate metabolism and the body levels of sodium and potassium, respectively.

Bile acids are mainly produced in the liver with use of cholesterol as the precursor. Bile acids assist the solubilization of dietary lipids by increasing the effective surface area of lipids. With more surface area, digestion of lipid by lipase and absorption by the intestine are more efficient.

1.1.4 Sources of cholesterol in our body

There are two sources of cholesterol in the body: endogenous and dietary (Kohlmeier, 2003). About 70% of cholesterol inside our body is synthesized endogenously. The major sites of production are liver and intestines. Dietary source of cholesterol provides the remaining 30% of body cholesterol. The largest source of cholesterol in our diet is eggs (4.3 mg/g). Other sources include organ meats, such as

liver (3.9 mg/g), and animal fats, such as lard (1.0 mg/g).

1.2 Lipid hypothesis

Lipid hypothesis (also called diet-heart hypothesis) was proposed by Keys in the late 1950's. The main idea of lipid hypothesis is that lowering blood cholesterol levels should significantly reduce the incidence of coronary heart disease (CHD). Many research works support this view, for example, Brown et al. (1990) conducted a clinical research that suggested low cholesterol in diet reduces the incidence of CHD.

1.2.1 Relationship between dietary cholesterol and plasma cholesterol

Dietary cholesterol had a strong linkage with plasma total cholesterol (TC). Higher dietary cholesterol intake can lead to a higher TC value. According to one U.S. study, the amount of cholesterol in the diet alters the amount in the serum by approximately 5 mg/dl for every 100 mg of change in diets (Brown, 1983). Hence, it is generally recommended to have a low cholesterol diet in order to prevent CHD. Egg is one of the dietary components that had been extensively investigated for its effect on blood cholesterol level in experimental animals. It is believed that egg intake is a risk factor of CHD because it is rich in dietary cholesterol and high serum cholesterol is a risk factor of CHD (Kritchevsky, 2004).

1.2.2 Hypercholesterolemia , atherosclerosis and coronary heart disease (CHD)

As mentioned above, dietary cholesterol can cause elevated TC. When the cholesterol concentration in plasma is higher than the benchmark, an individual is regarded as hypercholesterolemia. In human, plasma cholesterol level over 200mg/dL

is regarded as hypercholesterolemia (Perrault et al., 2000). Hypercholesterolemia is a risk factor of atherosclerosis. Atherosclerosis is the process of hardening and narrowing of blood vessels, which is caused by hypercholesterolemia. Figure 1.2 shows the stages leading to atherosclerosis. In brief, high cholesterol concentration in plasma causes the deposition of plaques in the arteries with the combination of cholesterol, other fatty substances, fibrous tissue, and calcium. Until the lipid materials accumulate to a certain level, more or less depends on individuals, plaque rupture results and causes blockages in the arteries (Deslypere et al, 1998; Schettlerballot et al., 1997; Stehbens et al., 1990). When the process occurs in brain, stroke occurs; while it occurs in major heart vessels, CHD occurs.

1.2.3 Individual variation

Not everyone has the same response to dietary cholesterol. Many studies demonstrated that some animals are hyper-responsive (sharp increase in TC) to dietary cholesterol while some are hypo-responsive (moderate increase in TC) (Beynen et al., 1987; Katan and Beynen, 1987; Jacob et al., 1983). However, the mechanisms for the differences in response to dietary cholesterol remain unclear. As plasma total cholesterol is associated with incidence of CHD, study on the mechanism why some animal are hyper-responsive while some are hypo-responsive to dietary cholesterol can help us better understand causes of CHD.

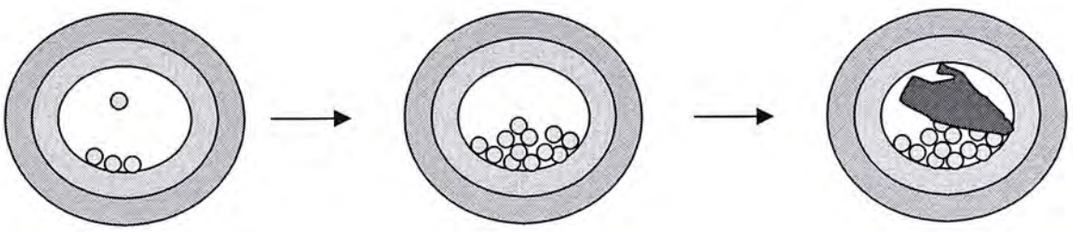

Figure 1.2. An illustration of the steps involved in atherosclerosis.

1.3 Cholesterol homeostasis

The major site controlling cholesterol homeostasis is liver, which synthesizes and contributes almost 70% of body cholesterol. The cholesterol homeostasis is maintained by three major mechanisms (Figure 1.3): (a) synthesis of cholesterol, which is controlled by a rate-limiting enzyme, 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) (Goldstein and Brown, 1984); (b) uptake of circulating blood cholesterol, by low-density lipoprotein receptor (LDL-receptor) (Brown and Goldstein, 1986) (c) elimination of cholesterol by converting it to bile acids, which is controlled by a rate-determining regulatory enzyme, cholesterol-7α-hydroxylase (CYP7A1) (Shefer et al., 1992). The expression of these regulatory proteins/enzymes is highly regulated, which under the control of transcription factors: SREBP-2 (Goldstein and Brown, 1999; 1997) and LXR-α (Gupta et al., 2002). SREBP-2 regulates the expression of LDL-receptor and HMG-CoA reductase while LXR-α up-regulates the gene expression of CYP7A1.

1.3.1 SREBPs up-regulates the expression of LDL-receptor and HMG-CoA reductase

Three isoforms of SREBPs have been identified: SREBP-1a, SREBP-1c and SREBP-2. SREBP-1a and SREBP-1c are produced from a single gene through the use of alternative transcription start sites encoding alternative first exons (Shimomura et al., 1997). A separate single gene encodes SREBP-2, which is 47% identical to SREBP-1a (Hua et al., 1993). Although SREBP-1a/c and SREBP-2 share similarity both in terms of amino acid sequences and structures, the functions of both are not identical. SREBP-1a/c plays an important role in fatty acid metabolism

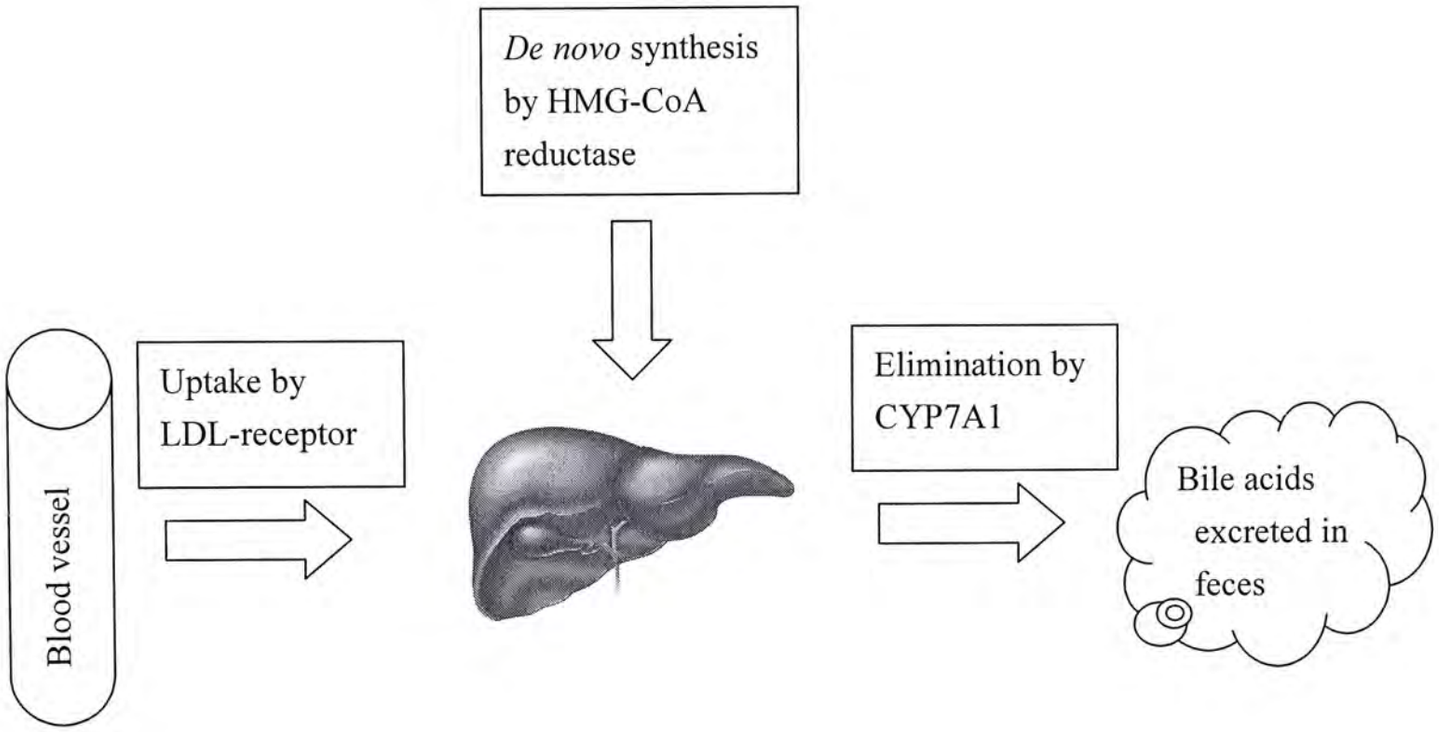

Figure 1.3. The homeostasis of cholesterol is controlled by liver, via three major mechanisms: uptake from blood, elimination in feces and endogenous biosynthesis.

whereas SREBP-2 plays a greater regulatory role in cholesterol metabolism.

SREBPs are synthesized as precursors bound to the endoplasmic reticulum (ER) and the nuclear envelop membrane in a hairpain orientation such that the N-terminal and C-terminal domains both face the cytoplasm (Osborne, 2000). The N-terminal domain SREBPs contains a basic helix-loop-helix leucine zipper (bHLH-ZIP) motif, which provides a DNA-binding domain. The precursor form of SREBPs cannot act as transcription factor until they are activated so that the N-terminal domain can translocate to the nucleus where it activates the transcription of sterol-responsive genes involved in cholesterol and fatty acid metabolism (Brown and Goldstein, 1997).

The activation mechanism of SREBP-2 has been most extensively studied. Release is accomplished by a two-step proteolytic cascade (Figure 1.4) that is regulated by sterols (Hua et al., 1996; Sakai et al., 1996; Wang et al., 1994). In sterol-depleted cells the cascade is initiated by a site-1 protease (S1P) that cleaves the middle of the luminal loop between two membrane-spanning domains (site-1 cleavage). Cleavage at site 1 breaks the covalent bond between the two transmembrane domains of SREBP-2, but both halves of the protein remain attached to membranes. Shortly thereafter, site-2 protease (S2P) clips the N-terminal of SREBP-2, which appears to reside within the first transmembrane domain (Sakai et al., 1996). This releases the active-form of SREBP-2 into the cytosol, from which it rapidly enters the nucleus and act as transcription factor.

SREBPs are believed as specific transcription factors that bind to 10bp sterol regulatory element (SRE) within the promoter region of the genes encoding the LDL receptor and HMG-CoA reductase (Vallett et al., 1996; Hua et al., 1993; Wang et al., 1993). Parallel expression of SREBPs and important proteins involved in cholesterol homeostasis suggests their important role in the regulation of sterol-responsive genes. Generally, when cellular cholesterol is low, SREBPs are activated and act as

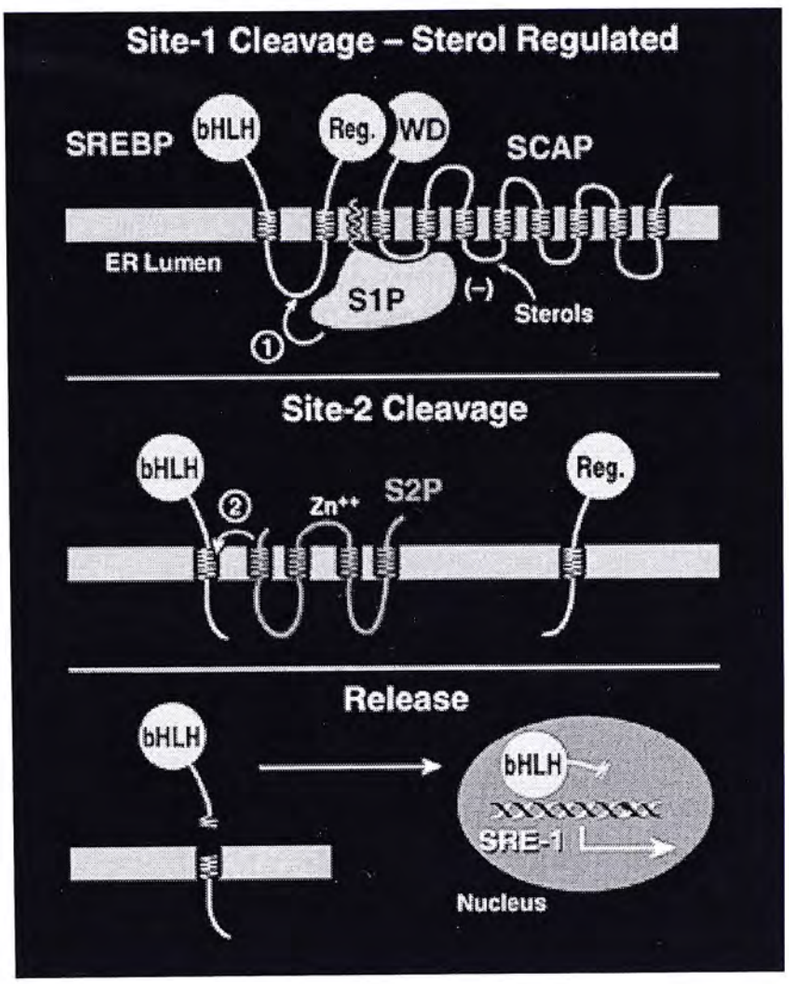

Figure 1.4. Model for the sterol-mediated proteolytic release of SREBPs from membranes. Adapted from Brown and Goldstein (1999).

transcription factors binding to genes encoding enzymes in the cholesterol biosynthesis pathway. In the study of Wang et al. (1993), it is suggested that LDL-receptor, which uptakes cholesterol from plasma LDL through receptor-mediated endocytosis, is regulated by SREBPs.

1.3.2 HMG-CoA reductase as the rate-limiting enzyme in cholesterol synthesis

HMG-CoA reductase (EC 1.1.1.34) is the rate-determining enzyme that involves in cholesterol biosynthesis (Brown et al., 1973). HMG-CoA reductase is an intrinsic membrane protein of the endoplasmic reticulum (ER) that catalyses the synthesis of mevalonate (Brown et al., 1974), which is a crucial intermediate in the formation of sterols and non-steroid isoprenoid compounds.

Cholesterol biosynthesis is controlled by the regulation of genes in response to intracellular cholesterol levels (Goldstein and Brown, 1990). When cellular cholesterol level is low, active forms of SREBPs (via proteolytic cleavage) are transported to the nucleus. The SREBPs then bind to SREs in the promoter regions of HMG-CoA reductase, thus increasing its transcription. In contrast, when cellular cholesterol level is high, the metabolites of cholesterol, such as hydroxyl-cholesterol, prevent the proteolytic cleavage of SREBPs to their active form, resulting in the down-regulation of HMG-CoA reducatase.

Since HMG-CoA reductase is responsible for the cholesterol biosynthesis in the body, it is believed that inhibition of this enzyme could help to lower blood cholesterol. Artificial HMG-CoA reductase inhibitors, such as lovastatin, pravastatin and simvastatin, are extensively used to cure patients with hypercholesterolemia. Some studies also suggest that water-soluble fibers can lower serum cholesterol concentration by inhibiting the activity of HMG-CoA reductase (Kay, 1982). The

mechanism for lowing blood cholesterol by inhibiting cholesterol biosynthesis was proposed by Brown and Goldstein (1986). When intracellular cholesterol synthesis is inhibited, more LDL-receptor is expressed to obtain cholesterol from blood for the needs in cells. Therefore, blood cholesterol is lowered by inhibiting HMG-CoA reductase.

1.3.3 LDL-receptor as the major protein removing plasma cholesterol

Removal of blood cholesterol is mediated by hepatic LDL-receptor, through a process called receptor-mediated endocytosis (Brown and Goldstein, 1986; 1984; Brown et al., 1981). Figure 1.5 shows the pathway for receptor-mediated endocytosis. LDL-receptors on cell-surface recognize the apolipoprotein (apo) B-100 on low density lipoprotein (LDL) and the complex is being internalized in a clathrin-coated pit that pinches off to become a coated vesicle. The vesicle containing the LDL rapidly loses its clathrin coat and fuses with other similar vesicles, forming larger vesicles called endosomes. LDL particle dissociates from LDL-receptors when fuse with late endosome, where the pH falls below 6 (Rudenko et al., 2002). The LDL-receptors are recycled by forming a separate vesicle and then travel back to the plasma membrane. The vesicle containing LDL particle fuses with the lysosome where lysosomal hydrolases degrade the apo-B protein to amino acids and release the cholesterol in the cellular region (Goldstein et al., 1975). The released free cholesterol is used for the production of bile acids, steroid hormones and the cell membrane.

The regulation of LDL-receptors is supposed to be at transcription level (Goldstein and Brown, 1990), in response to cellular free cholesterol concentration. As mentioned in the section 1.3.1, promoter of LDL-receptor gene contains an SRE, which is the binding site for SREBPs. Low level of cellular cholesterol induces the activation of SREBP-2, which acts as transcription factor, and up-regulate the

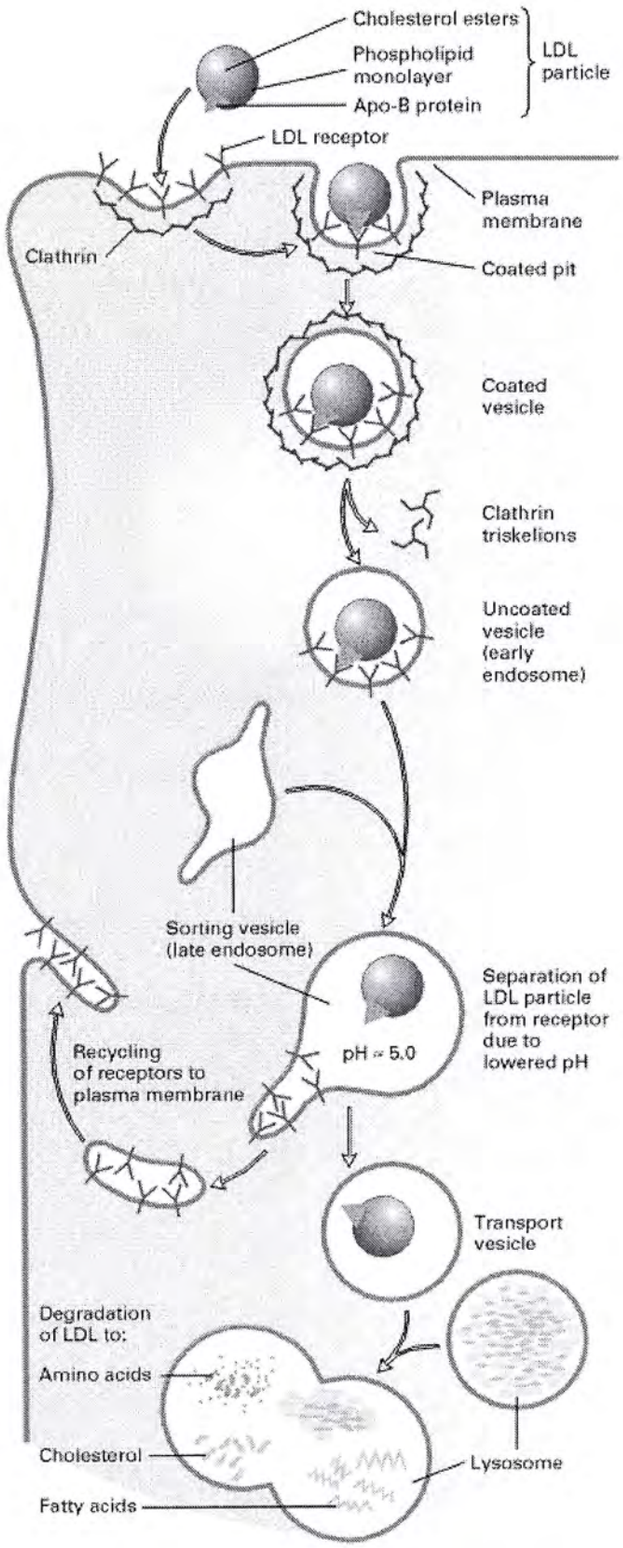

Figure 1.5. Receptor mediated endocytosis pathway of LDL-receptor. Adapted from Lodish et al. (2000).

transcription of LDL-receptor gene (Hua et al., 1996). Similar finding was observed in human mononuclear cells that cholesterol-restricted diet caused an up-regulation of LDL-receptor mRNA expression (Boucher et al., 1998). Hence, the up-regulation of LDL-receptor, at transcriptional level, seems to be a compensatory mechanism for the cholesterol deficiency.

1.3.4 LXR-α as an activator of CYP7A1

LXR-α is expressed mainly in the liver, whereas lower concentrations are expressed in the kidney, intestine, spleen and adrenals (Willy et al., 1995). LXR-α was first identified as orphan nuclear receptors. They consist of a central DNA-binding domain and a hydrophobic C-terminal domain that mediates ligand recognition, receptor dimerization and ligand-dependent activation (Peet et al., 1998a). LXR-α is activated by naturally occurring oxysterols (Janowski et al., 1996).

Figure 1.6 shows the process of CYP7A1 up-regulation by LXR-α. To function as transcription factors, LXR-α has to heterodimerize with the retinoid X receptors (RXRs). This heterodimer can be activated by ligands for either RXR or LXR. Binding of ligands to LXR response element (LXRE) in the promoter region induces the conformational changes in the LXR-RXR heterodimer, resulting in enhanced transcription of a target gene (Willy and Mangelsdorf, 1997). The parallel expression pattern of LXR-α and oxysterols indicates LXR-α is involved in cholesterol metabolism. For example, up-reguation of LXR-α is associated with enhanced CYP7A1 expression (Gupta et al., 2002; Chiang et al., 2001).

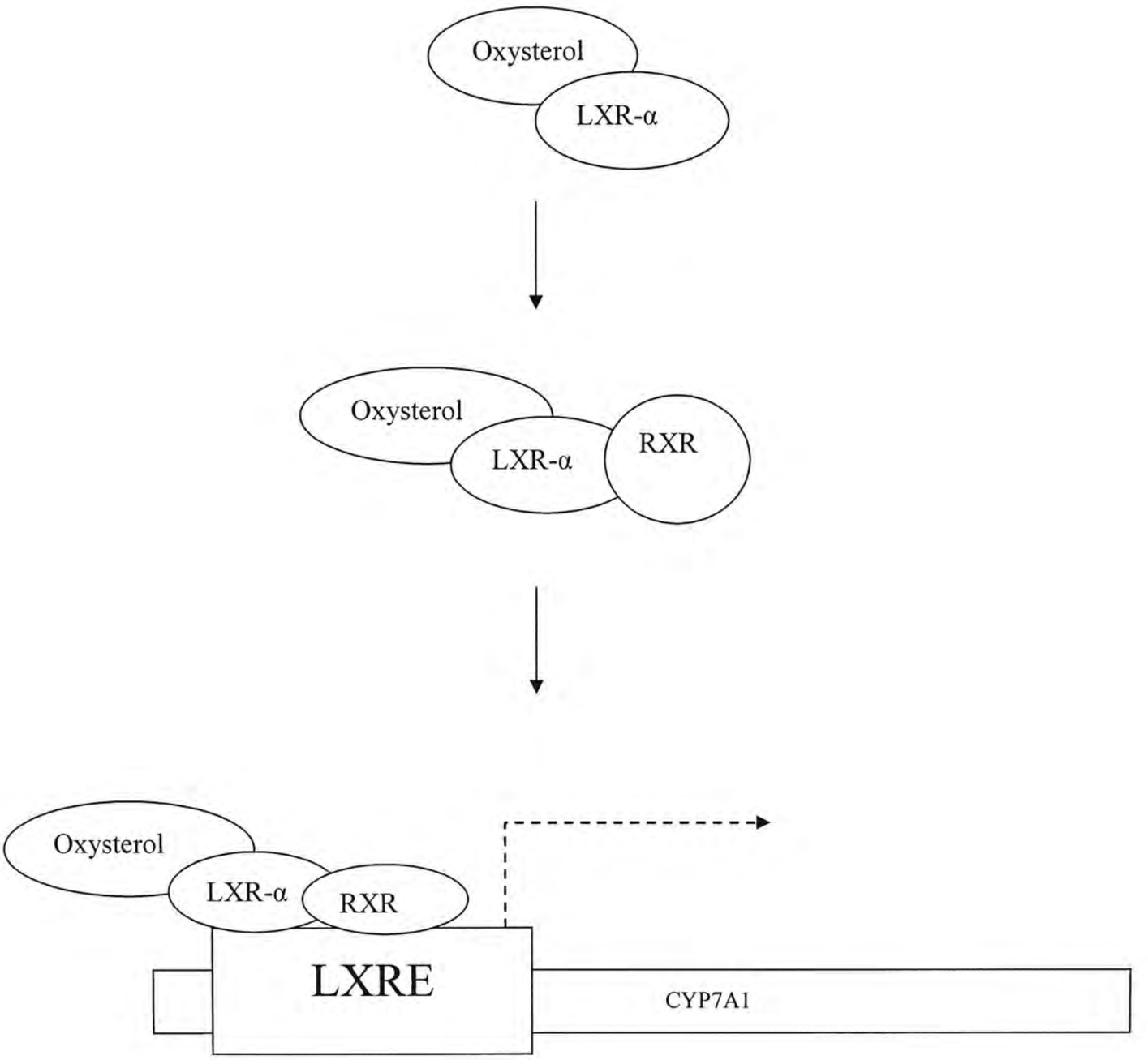

Figure 1.6. LXR-α up-regulates the transcription of CYP7A1 by forming heterodimer with RXR and binding to the LXRE of CYP7A1.

1.3.5 CYP7A1 controls the classical pathway for the elimination of hepatic cholesterol

CYP7A1 (EC 1.14.13.17) controls the first and rate-limiting step on the catabolic pathway from cholesterol to bile acids. It catalyzes the conversion of cholesterol to 7α-hydroxycholesterol by addition of a hydroxyl group on the C-7 position on cholesterol. Subsequently, 7α-hydroxycholesterol is modified by more than 17 enzymes to convert into primary bile acids (Russell, 2003). As bile salts are the major excretory products of cholesterol catabolism (Russell and Setchell, 1992), CYP7A1 plays an important role in the removal of cholesterol from cell and from the blood stream.

CYP7A1 is regulated positively by cholesterol in the liver and negatively by bile acids. In response to increased dietary cholesterol, the hepatic cholesterol pool is maintained by an increased conversion of cholesterol to bile acids, via CYP7A1 pathway, or secretion of free cholesterol into bile. It is interesting to note that the extent of bile acid synthesis responding to dietary cholesterol varies among species. Dietary cholesterol increases mRNA and activity of CYP7A1 as a compensatory response to maintain cholesterol homeostasis in rats (Shefer et al., 1992; Pandak et al., 1991). In contrast, repressed mRNA expression and activity of CYP7A1 were observed in hamsters feeding cholesterol (Horton et al., 1995). Negative feedback of bile acid in CYP7A1 has been demonstrated by many studies. Early study by Bergstrom and Danielsson (1958) has showed that injection of bile acids into rats caused a rapid reduced rate of bile acids excretion. Subsequent studies by Shefer et al. (1970; 1969) have directly indicated that the reduced bile acids excretion after injection of taurocholate is due to the blockage of 7α–hydroxylation of cholesterol, which is a process controlled by CYP7A1. In contrast, increased CYP7A1 mass,

transcription and activity was observed in biliary diverted rats, where bile acids reabsorption was inhibited (Parks et al., 1991). Hence, the difference in the extent of bile acid synthesis in response to dietary cholesterol in different species may be due to different level of bile acids return to the liver, which exerts different level of negative inhibition on CYP7A1.

Clinically, bile acid sequestrants are widely used in the treatment of hypercholesterolemia. Two commonly used bile acid sequestrants are cholestyramine and colestipol (Wilson et al., 1998). Bile acid sequestrant, such as cholestyramine, acts by binding of bile acids in the duodenum, and carried to the ileum and then excreted in feces. Diminished pool of bile acids prompts the conversion of more hepatic cholesterol into bile acids, by the action of CYP7A1 mentioned above. As a result of depletion of liver cholesterol, increased hepatic LDL-receptor activity and enhanced LDL clearance from circulation helps in lowering plasma cholesterol levels (Ast and Frishman 1990; Packard and Shepherd, 1982).

1.3.6 Bile acids as the metabolites of CYP7A1

As mentioned in 1.1.3, bile acids are important metabolites in eliminating cholesterol from our body. There are two distinct pathways for the bile acid synthesis: the classical (neutral) and the alternative (acidic) pathway. Microsomal cholesterol 7α-hydrolyase (from CYP7A1 gene) is the rate-limiting enzyme in the classical bile acid synthesis, and mitochondrial sterol 27-hydroxylase is suggested to be the limiting enzyme in alternative bile acid synthesis. The major pathway of bile acids biosynthesis is the classical pathway, which constitute about 80% of the bile acids excreted (Russell and Setchell, 1992).

The catabolism of cholesterol is a process of conversion of cholesterol from a

highly water-insoluble lipophilic molecule to a smaller hydrophilic bile acid. The conversion process involves multiple hydroxylations of cholesterol structure by a series of at least 17 metabolic intermediary enzymatic steps (Russell, 2003). The two primary bile acids, chenodeoxycholic and cholic acids, are the most abundant in the bile. They are produced by hydroxylations at C-7 or at both C-7 and C-12 on cholesterol (Figure 1.7). These two bile acids are synthesized in equal amounts via classical (neutral) pathway involving the rate-limiting enzyme, CYP7A1 (Russell, 2003). Bacterial reactions in the intestine convert primary bile acids into secondary bile acids. Cholate and chenodeoxycholate are converted to deoxycholate and lithocholate, respectively. In humans, approximately 95% of both primary and secondary bile acids are reabsorbed by the intestines and delivered back to the liver via the portal circulation, in the process called enterohepatic circulation. This process plays an important physiological role in lipid absorption, bile acid synthesis and cholesterol homeostasis.

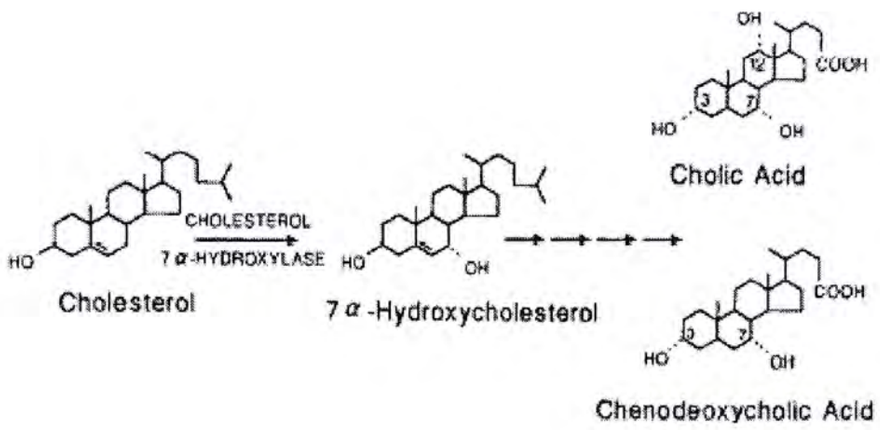

Figure 1.7. CYP7A1 controls the conversion of cholesterol to 7α-hydroxycholesterol. Modified from Jelinek et al.(1990).

1.4 Previous works in our laboratory

It is known that each population has some individuals who are resistant to diet-induced hypercholesterolemia and atherosclerosis (Herron et al., 2003; Jacobs et al., 1983). This phenomenon has been found also in different animal species (Gao et al., 2002; Sullivian et al., 1993). For example, Golden Syrian (GS) hamsters are hyper-responders to dietary cholesterol while guinea pigs and Sprague Dawley (SD) rats are hypo-responders to high cholesterol diets.

In order to establish the animal models for studying the mechanisms of hypo- or hyper-repsonsive effect to dietary cholesterol, effect of dietary eggs on serum cholesterol level in both rats and hamsters were previously studied in this laboratory. Interestingly, supplementation of boiled egg in diet (1-2 eggs/2000 kcal or 1.1-1.7 g cholesterol/kg diet, equivalent to 1-2 eggs/day/per person in humans) did not raise but instead it lowered cholesterol level in SD rats by 24-32%; whereas in hamsters, egg supplementation at the same level in diet significantly elevated serum cholesterol level by 19-42% (Figure 1.8). These results clearly suggest that two species respond very differently to a high cholesterol diet. The observation arouses our interest to investigate the underlying mechanisms. It is believed that effect of dietary cholesterol on its serum level is a complex process that involves multifaceted dietary cholesterol-gene interaction.

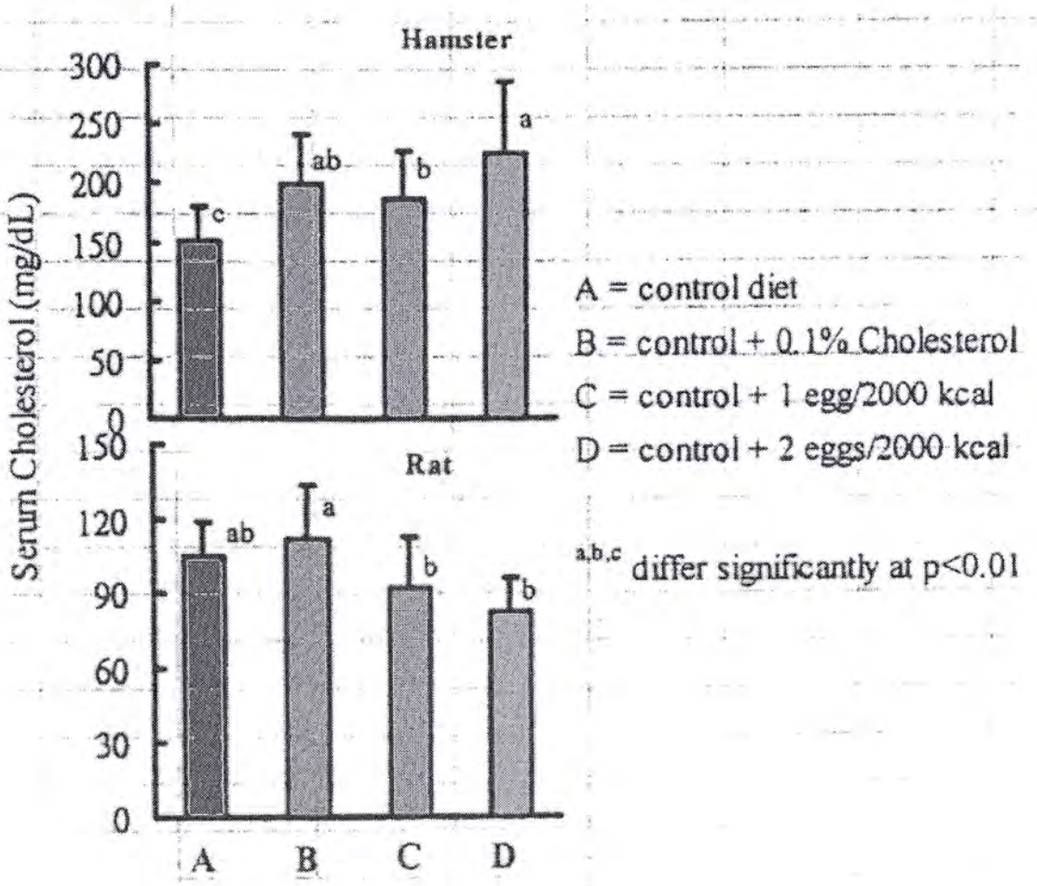

Figure 1.8. Effect of egg supplementation in diet on serum cholesterol level in rats and hamsters (n = 12). Unpublished data in our laboratory.

1.5 Objective of this project

This study focused on how the expression pattern of the above mentioned regulatory proteins and its transcription factors in two animal models (SD rats and GS hamsters) lead to the different response to dietary cholesterol.

There are two parts in this project. For the first part of experiment, whether there are any differences of proteins/enzymes expression profile in response to high cholesterol diets was determined. Rats and hamsters, which act as a hypo-responsive and a hyper-responsive model respectively, were chosen in the present study. In addition, the level of regulation (transcription or translation) of those important proteins/enzymes was determined. The first part of experiment could help us understand how the difference in protein/enzyme expression led to different response to a high cholesterol diet.

The second part of the experiment was to establish the relationship of serum cholesterol and the expression level of proteins related to cholesterol homeostasis. For example, whether higher LDL-receptor expression in some individual leads to a lower serum cholesterol. This part of experiment could help explain why some individuals in the same species are hyper-responsive while some are hypo-responsive to a high cholesterol diet.

CHAPTER 2

Increased expression of LDL-receptor is responsible for the hypo-responsiveness of rats to dietary cholesterol

2.1 Introduction

Hepatic cholesterol is derived from the *de novo* biosynthesis, which is controlled by HMG-CoA reductase (Goldstein and Brown, 1984), and receptor-mediated uptake of plasma cholesterol, by the action of LDL-receptor (Brown and Goldstein, 1986). LDL-receptors provide hepatocytes with a highly efficient means for supplying adequate amount of readily available cholesterol. LDL-receptor recognize apo-B 100 of plasma LDL and uptake them into the liver, via a process called receptor-mediated endocytosis (Brown and Goldstein, 1986). HMG-CoA reductase, a rate-determining enzyme for the biosynthesis of cholesterol, provides endogenous source of cholesterol when the cellular level of cholesterol is low. It has been demonstrated that LDL-receptor and HMG-CoA reductase mRNA are coordinately regulated (Rudling,1992). Subsequent studies have shown that the coordination between LDL-receptor and HMG-CoA reductase are under the control of SREBP-2 (Goldstein and Brown, 1990). When cellular cholesterol level is low, more SREBP-2 is converted from precursor form to nuclear form, which binds to the promoter region and up-regulate LDL-receptor and HMG-CoA reductase. It has been postulated that different responsiveness to dietary cholesterol in animals is, at least partially, due to the different expression level of LDL-receptor and HMG-CoA reductase in response to high cholesterol diet.

2.2 Objective

Rats were used as a "hypo-responsive" model where hamsters were a "hyper-responsive" model to dietary cholesterol in this study. This study was performed to determine the differences in the cholesterol uptake and biosynthesis in the liver of both animals in the response to a high cholesterol diet. In addition, the mechanism for the differences in the protein/enzyme was also determined.

2.3 Methods and materials

2.3.1 Animals

Sprague-Dawley (SD) rats and Golden Syrian (GS) hamsters were housed in cages in a room with controlled temperature (20 – 23℃) and lighting (alternating 12 hour periods of light and dark). Prior to the studies, the animals were fed a pelletized commercial nonpurified diet for 6 days after arrival. The body weight of rats was 190 to 240 grams while that of the hamsters was 120 to 150 grams.

The animals were then randomly divided into 4 groups (n = 5) and fed a semisynthetic diet (control diet) with or without supplementation of 0.3%, 0.6% or 0.9% (wt/wt) cholesterol (Sigma Chemical Co. St. Louis, MO) *ad libitum* for 4 weeks. All studies were carried out during the mid-dark phase of the light cycle. The animals were killed in a nitrogen gas tank. Liver, white adipose tissue (epididymal), brown adipose tissue (interscapular), kidney cortex (separated from the renal medulla), heart, skeletal muscle and brain were rapidly removed and frozen in liquid nitrogen.

2.3.2 Diets

The control semisynthetic diet used in these studies contained (wt/wt) 10% lard, 30.5% corn starch, 25.7% sucrose, 24.9% casein, 3.7% AIN-76 mineral mix, 1.1% AIN-76 vitamin mix, 0.4% choline bitartrate, 0.3% DL-methionine and 3.4% cellulose. The ingredients were purchased from Harlan Teklad (Madison, WI) except for lard, which was obtained from the local market, and DL-methionine and cholesterol, which were purchased from Sigma Chemical (St. Louis, MO).The experimental diets were prepared by supplementing the control semisynthetic diet (0.0%) with 0.3%, 0.6% and 0. 9% cholesterol.

2.3.3 Determination of serum cholesterol

Serum total cholesterol (TC) levels was determined enzymatically by the use of commercial kits (Sigma Chemical, St Louis, MO, USA).

2.3.4 Western blot

The protein was extracted according to the method described by Vaziri et al. (1996) with some modifications. Briefly, frozen rat liver was homogenized in lysis buffer with 20mM Tris-HCl (pH 7.5) containing 2mM $MgCl_2$, 0.2M sucrose and protease inhibitor cocktail pellet (Complete, Roche, Mannheim, Germany). The crude extract was separated into two aliquots. One was centrifuged at 12,000 rpm for 15 minutes at 4℃. The supernatant was considered as total protein fraction. Another aliquot was centrifuged at 3,000 rpm for 10 minutes at 4℃. The supernatant was then centrifuged at 35,000 rpm for 60 minutes at 4℃. The solubilized pellet was considered as membrane protein fraction. Protein concentration of two fractions was determined by using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, California, USA).

For the measurement of LDL-receptor and HMG-CoA reductase, the membrane protein (100μg) was size-fractionated on 8 % SDS-PAGE at 120V for two hours. After electrophoresis, proteins were transferred to a Hybond-ECL membrane (Amersham Life Science Inc., Arlington Heights, IL, USA). The membrane was incubated for one hour in blocking solution (1x TBS, 0.1% Tween-20 and 5% nonfat milk) at room temperature and then overnight at 4℃ in the same solution containing 1:300 anti-LDL receptor antibody (Santa Cruz Biotechnology, Inc., California, USA) or 1:500 anti-HMG-CoA reductase (Upstate USA Inc., Lake Placid, NY, USA) whichever appropriate. Membrane was washed once for 15 minutes then twice for

five minutes in washing solution (1x TBS and 0.1% Tween-20) prior to one hour incubation in blocking solution containing diluted (1:3000) horseradish peroxidase-linked rabbit anti-goat IgG (Zymed Laboratories Inc., South San Francisco, USA) or donkey anti-rabbit IgG (Amersham Life Science Inc., Arlington Heights, IL, USA). The washes were repeated before the membranes were developed with chemiluminescent agents (ECL; Amersham Life Science) and subjected to autoradiography for one to five minutes.

For SREBP-2, the membrane protein (100μg) and total protein (50μg) aliquots were size-fractionated on 8% SDS-PAGE at 120V for two hours. The whole procedures were described as above except for 1:300 anti-SREBP-2 antibody (Santa Cruz Biotechnology, Inc., USA) was used as primary antibody. Both precursor and mature form of SREBP-2 were identified on the same membrane.

2.3.5 Probe production for LDL-receptor

2.3.5.1 Extraction of total RNA

RNA was isolated from frozen liver using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Briefly, 100mg of liver was homogenized in 1mL of TRIzol reagent. After that, 0.2mL of chloroform was added and centrifuged at 12,000 rpm for 15 minutes at 4℃. The supernatant was transferred to a clean eppendorf containing 0.5mL isopropanol. The mixture was centrifuged again at 12,000 rpm for 15 minutes at 4℃. The supernatant was removed and 70% ethanol was added to wash the pellet. It was then centrifuged at 8,000 rpm for 10 minutes at 4℃. The supernatant was removed and the pellet was dissolved with formamide. The RNA concentration was determinutesed from the absorbance at 260nm and all samples had an 260/280 nm absorbance ratio of about 2.0.The extracted RNA was stored at -70℃ until further use.

2.3.5.2 Reverse-transcription reaction of total RNA

The reverse-transcription was carried out by SuperScript™ II RT (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, 1μg of total RNA was added to reaction mixture contained Oligo(dT)12-18 (500μg/ml), 1μl dNTP Mix (10mM each) 1μl and the final volume was adjusted to 12μl by autoclaved double distilled water. The reaction mixture was heated to 65℃ for 5 minutes and chilled on ice quickly. After that, First-Strand Buffer (1x) and DTT (0.1 M) was added to the mixture and the mixture was heated again at 42℃ for 2 minutes. After heating, 1μl (200 units) of SuperScript™ II RT was added and incubated at 42°C for 50 minutes. Finally, the reaction was inactivated by heating at 70°C for 15 minutes. The cDNA was used as a template for amplification in PCR.

2.3.5.3 Polymerase chain reaction (PCR) of LDL- receptor fragment from cDNA template

The LDL-receptor sequence was amplified using Taq DNA polymerase (Gibcol BRL, Grand Island, NY, USA) from the cDNA template. Forward primer (5' – att ttg gag gat gag aag cag – 3') and reverse primer (5' – cag ggc ggg gag gtg tga gaa – 3') (Life Technology, USA) were designed (size: 931bp) based on the sequence published in GeneBank database (assession no.: X13722).

The PCR reaction mixture contained 1μl first cDNA, 1x PCR buffer, 1.5mM $MgCl_2$, 0.2mM of each dATP, dCTP, dGTP, dTTP, 1.0μM primers and 1U Taq DNA polymerase (Gibcol BRL, Grand Island, NY, USA), the final volume was adjusted to 50μl by autoclaved double distilled water. Reaction mixtures were heated to 94℃ for 5 minutes to denature the template completely before addition of Taq polymerase. PCR reactions were performed in Peltier Thermal Cycler PTC200 (MJ Research Inc., Waltham, Massachusetts, USA) for 25 cycles and the condition was 94℃ for 1

minutes, 60℃ for 1 minutes and 72℃ for 2 minutes. After completing 25 cycles, the reaction mixture was heated at 72℃ for 10 minutes and was kept at 4℃ until use.

2.3.5.4 Separation and purification of PCR products

PCR products were separated on 1% agarose gel (Gibcol BRL, Grand Island, NY, USA) containing ethidium bromide in TAE buffer (40mM Tris, 20mM Sodium Acetate, 1mM EDTA, pH 7.4) by electrophoresis. PCR products were visualized under UV light and bands of correct size, determined by comparison with DNA molecular weight markers (Gibcol BRL, Grand Island, NY, USA), were cut out by ordinary razor blade.

DNA excised from the agarose gel was purified by GeneClean II (Bio101, La Jolla, CA, USA) according to the manufacturer's protocol. Briefly, 1 ml of sodium iodide solution was added to the agarose gel with DNA band and incubated at 55℃ until the gel was dissolved completely. 5μl of GLASSMILK suspension from the kit was then added to the above mixture solution. The reaction mixture was kept on ice with constant agitation for about 20 minutes to allow DNA bind to the GLASSMILK beads. The reaction was terminated by brief centrifugation to spin-down the beads. The supernatant was then discarded and the pellet was washed three times with New Wash Solution supplied together with the kit. The washed pellet was re-suspended in 20μl autoclaved double distilled water and was incubated at 55℃ for 10 minutes. Finally, the GLASSMILK beads were spun down for 1 minute and the supernatant containing the eluted DNA was collected and stored at -20℃ until use.

2.3.5.5 Polishing of purified PCR products

PCR fragment were polished in order to create the blunt ends needed to improve overall cloning efficiency. The procedures were carried out according to the PCR-Script^{TM} Cloning Kit (Strategene, La Jolla, CA, USA) Briefly, 1mM dNTP mix,

1μl 1x polishing buffer and 0.5U of cloned pfu DNA polymerase were added orderly to the purified PCR products and the final volume was adjusted to 10μl by autoclaved double distilled water. The reaction mixture was incubated for 30 minutes at 72℃. The polished PCR products were stored at -20℃ until further use.

2.3.5.6 Ligation of PCR products and pPCR-script Amp SK(+) cloning vector

The PCR products were inserted into the cloning vector by PCR-Script™ Cloning Kit (Strategene, La Jolla, CA, USA) according to the manufacturer's protocol. Briefly, ligation mixture containing 10ng pPCR-Script Amp SK(+) cloning vector, 1x PCR-Script reaction buffer, 0.5mM rATP, polished PCR products, 5U Srf I restriction enzyme, 4U T4 DNA ligase were mixed and the final volume was adjusted to 10μl by autoclaved double distilled water. The ligation reaction was carried out for one hour at room temperature and stopped by heating the reaction mixture for 10 minutes. The ligation reactions were stored on ice before transformed into DH5α competent cell.

2.3.5.7 Transformation

Transformation was performed according to the manufacturer's protocol. Briefly, 3μl of ligation mixture was added to 100μl DH5α competent cell. The mixture was swirled gently and incubated on ice for 30 minutes for the plasmid DNA attached to the cell surface of the competent cells. The mixture was subjected to heat shock at 42 ℃ for 40 seconds to allow the plasmid enter the host cell and then chilled on ice immediately for 2 minutes. Thereafter, the competent cell and plasmid mixture was incubated in 1ml LB medium at 37℃ for 1 hour with shaking at 225-250 rpm to allow cells grow. The LB medium was subsequently centrifuged for 5 minutes to pellet the cells. The cell pellet was dissolved in 100μl LB medium. 40μl of 2% X-gal and 4μl of IPTG (200mg/ml) were added to the resuspended cells in order to allow blue-white color screening. The final transformation mixture was spread evenly on a

pre-warmed agar plate with 50μg/ml amplicillin. The plates were incubated overnight at 37℃ to allow growth of colonies.

2.3.5.8 Preparing glycerol stocks containing the bacterial clones

Several white colonies were picked from each of the overnight-cultured agar plates by sterilized pipette tips. Each colony was grown in 10ml Amp-LB medium at 37℃ overnight with shaking at 250 rpm to allow the growth of bacteria containing the plasmids. After that, 500μl of the overnight-cultured bacterial clone was pipetted out and transferred to a sterile 1.5ml tube containing equal volume sterile 80% glycerol (Sigma, St Louis, MO, USA). The mixture was mixed thoroughly and kept at -70℃ for long-term storage.

2.3.5.9 Plasmid DNA preparation

The plasmid DNA was extracted from the overnight-cultured Amp-LB medium containing the bacterial clones by Wizard ® Plus SV Minipreps DNA Purification Sysrem (Promega, Madison, Wis., USA) according to the manufacturer's protocol. Briefly, medium containing the bacterial clones were pelleted by centrifugation for 15 minutes at 3,500 rpm. The supernatant was discarded and the pellet was resuspended with 250μl cell resuspension solution. 250μl cell lysis solution and 10μl alkaline protease solution were added to each sample and mixed. The reaction mixture was incubated at room temperature for 5 minutes. After that, 350μl neutralization solution was added and the reaction mixture was centrifuge at 12,000 rpm for 10 minutes at room temperature. The cleared lysate was decant into spin column and centrifuged at 12,000 rpm for 1 minute at room temperature and the flowthrough was discarded. After addition of 750μl wash solution, the reaction mixture was centrifuged at 12,000 rpm for 1 minute and the flowthrough was discarded. The previous step was repeated with 250μl wash solution. 100μl nuclease-free water was added to the spin column

and the whole set up was centrifuged at 12,000 rpm for 1 minute. The eluted water containing plasmid DNA was kept in eppendorf and stored at -20℃ until use.

2.3.5.10 Clones confirmation by restriction enzyme digestion

To check whether the clones contained the LDL-receptor fragment, the DNA plasmid was digested by restriction enzymes. Insert in the cloning vector was released by cutting the plasmid with restriction enzymes, Hinc II (Gibcol BRL, Grand Island, NY, USA). Restriction enzyme reaction mixture contained plasmid DNA, 10U of enzyme and suitable buffer. The reaction was carried out at 37℃ for 1 hour. The reaction mixture was then separated by electrophoresis on the agarose gel and the size of the released fragments was compared with the DNA molecular markers. The uncut plasmid DNA was also loaded on the gel as control. A fragment, which was about 300bp was released after the enzyme digestion. Clones that could generate fragments with correct size were selected out and performed automatic sequencing for further confirmation.

2.3.5.11 Clones confirmation by automatic sequencing

Automatic sequencing was used to further check whether the clones contained LDL-receptor fragment. This was performed using ABI-PRISM^{TM} DNA sequencing kit (Perkin Elmer, Foster City, California, USA) in an ABI-PRISM^{TM} DNA sequencing system (Advanced ABI 310 Genetic Analyzer, Perkin Elmer, Foster City, California, USA) according to the manufacturer's protocol.

The sequencing mixture contained 50ng plasmid DNA, 3.2pmole of primer (either upstream or downstream), 8μl Terminator Ready Reaction Mix (Perkin Elmer, Foster City, California, USA) and the final volume was adjusted to 20μl by autoclaved double distilled water. Reaction was carried out in a DNA thermal cycler for 25 cycles under the following condition: 96℃ for 1 minute, Tm of the primer

used for 1 minute and 60℃ for 4 minutes. Upon completion, the reaction mixture was precipitated by 2 volumes of absolute ethanol and 0.1 volume of 0.5M sodium acetate at pH 5.4 at -70℃ for 1 hour and pelleted by centrifugation at 12,000 rpm for 30 minutes at 4℃. The pellet was washed with 70% ethanol and ethanol was evaporated. After the last trace of ethanol was evaporated, the pellet was dissolved in 20μl autoclaved doubled distilled water. Equal volume of Terminator Ready Reaction buffer supplied with the sequencing kit was added to the dissolved DNA. The mixture was mixed and incubated at 95℃ for 3 minutes. Immediately after that, the mixture was chilled for 2 minutes. Finally, the content was analyzed using an automated DNA sequencer.

The sequencing data obtained were submitted to the BLAST sequence similarity search (NCBI, National Center for Biotechnology Information) to confirm whether the clone contained target sequence. A typical profile of automatic sequencing was showed in Figure 2.1.

2.3.5.12 Linearization of the plasmid DNA

The plasmid DNA template that contained the LDL-receptor fragment was linearized by restriction enzyme, Hind III, which had one cutting site between the T7 promoter region and the 5' end of the insert. The reaction mixture contained more than 1μg of plasmid DNA, 1x appropriate buffer for the restriction enzyme, 1U of Hind III (Gibcol BRL, Grand Island, NY, USA) and the final volume was adjusted to 20μl by autoclaved double distilled water. After the reaction was completed, 0.1 volume of the digested mixture was pipetted out and loaded on 0.1% agarose gel to ensure that all plasmids had been linearlized. The remaining linearized plasmid DNA was extracted once by phenol/chloroform and precipitated by ethanol precipitation. The reaction mixture was precipitated by 2 volumes of absolute ethanol and 0.1 volume of 0.5M

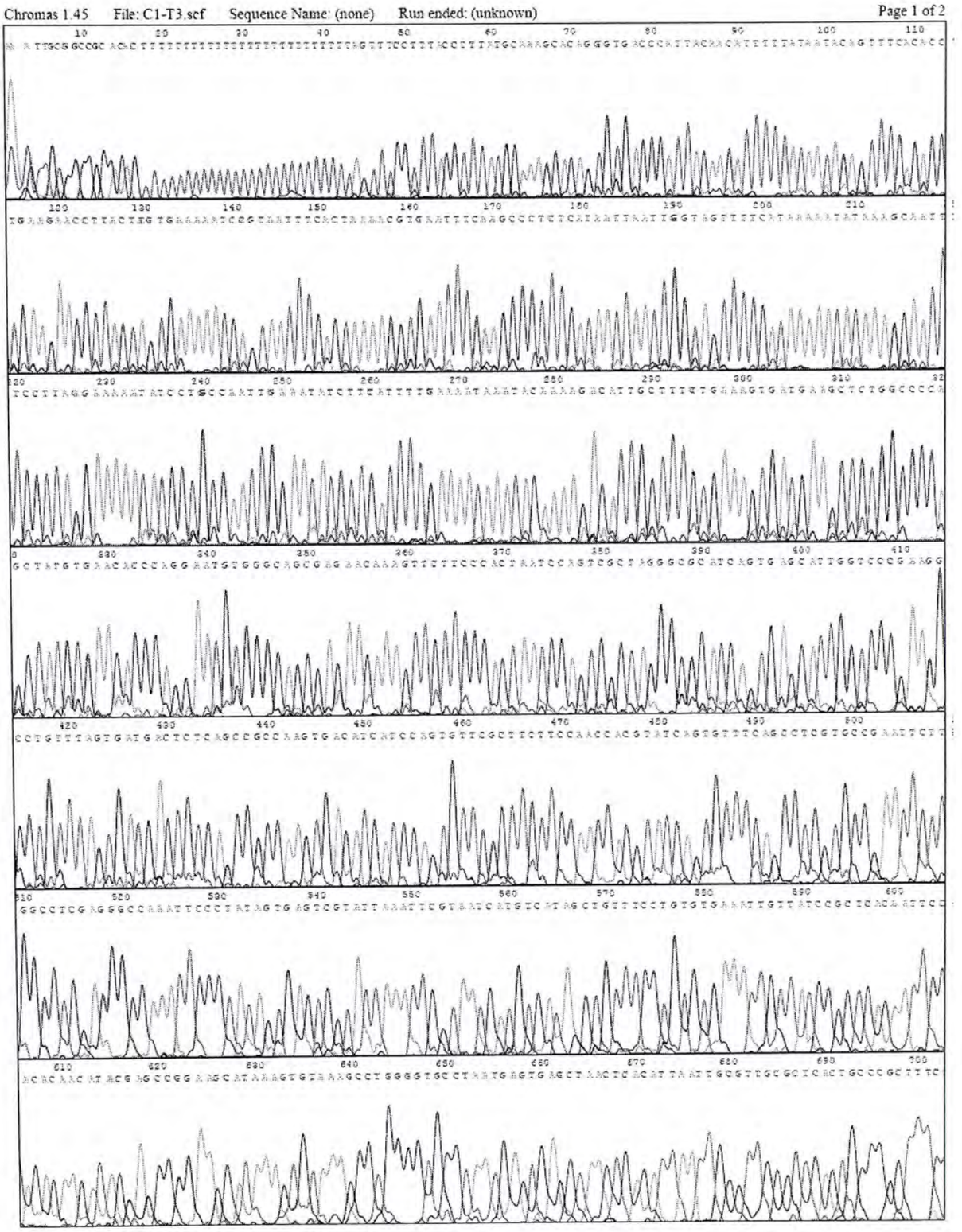

Figure 2.1. A typical profile of automatic sequencing for the confirmation of clones in DNA/RNA probe production.

sodium acetate at pH 5.4 at -70℃ for one hour and pelleted by centrifugation at 12,000 rpm for 30 minutes at 4℃ and washed in 70% ethanol. After the evaporation of the last trace of ethanol, the pellet was dissolved in 20µl autoclaved double distilled water.

2.3.5.13 DIG-labeling of RNA probe

DIG RNA labeling kit (Roche, Mannheim, Germany) was used for the production of DIG-labeled RNA probe. DIG-RNA labeling reaction was performed according to the manufacturer's protocol. Briefly, 1µg of linearized and purified LDL-receptor template was added to a sterile, RNase-free microfuge tube containing 2µl of 10x NTP labeling mixture, 1µl of RNase inhibitor, 2µl of 10x transcription buffer and 2µl of T3 RNA polymerase (Roche, Mannheim, Germany). The final volume was filled up to 20µl by adding DEPC-treated water. The reaction mixture was mixed gently and centrifuged briefly to collect all the mixture at the bottom of the tube. It was then incubated at 37℃ for 2 hours. To remove DNA template in the reaction mixture, 2µl of DNase I was added and incubated at 37℃ for additional 15 minutes. 2µl of 0.2M EDTA was then added to stop the transcription reaction. To purify the DIG-labeled RNA probe from the reagents, 0.1 volume of 4M LiCl, 3 volume of cold absolute ethanol was added to the reaction mixture. It was gently mixed and incubated at -70℃ for 1 hour. After incubation, the tube was centrifuged at 13,000 rpm for 30 minutes at 4℃ and the RNA pellet was washed by 70% ethanol. The DIG-labeled LDL-receptor probe was then dissolved in 100µl DEPC-treated water with 1µl RNase-inhibitor. The probes were aliquoted into 10 microfuge tubes each containing 10µl solution and stored at -70℃ until use.

2.3.5.14 Testing of DIG-labeled probe

Before performing Northern hybridization, it was essential to test whether the

RNA probe had been properly DIG-labeled. This was done according to the manufacturer's protocol. Briefly, 2μl of newly synthesized DIG-RNA probe was spotted on a small piece of nitrocellulose membrane. RNA probe was fixed on the membrane by UV cross-linking. The membrane was washed briefly in maleic acid buffer (0.1M Maleic acid, 0.15M NaCl, pH 7.5) for 5 minutes at room temperature, followed by the incubation of membrane in 1x blocking solution (Roche, Mannheim, Germany) for 15 minutes. Anti-DIG antibody diluted with 1x blocking solution (1:1500) was added to the membrane and incubated for 15 minutes. The membrane was washed in maleic acid buffer for 3 times, 5 minutes each. Then, the membrane was washed in maleic acid buffer with 0.05% Tween-20 for 3 minutes. To detect the desired spots on the membrane, colour-substrate solution (NBT/BCIP, Roche, Mannheim, Germany) dissolved in 10ml of DEPC-treated water was added and incubated at room temperature until colour was observed. Dark purple spot appeared on the membrane represented that the RNA was labeled and could be used for Northern hybridization.

2.3.6 Probe production for HMG-CoA reductase

The expressed sequence-tagged (EST) clone for HMG-CoA reductase (Image ID: 1 771 090) was purchased from ATCC (VA, USA). Several white colonies were picked from each of the agar plates supplied from ATCC by sterilized pipette tips. Each colony was grown in 10ml Amp-LB medium at 37℃ overnight with shaking at 250 rpm to allow the growth of bacteria containing the plasmids. After that, 500μl of the overnight-cultured bacterial clone was pipetted out and transferred to a sterile 1.5ml tube containing equal volume sterile 80% glycerol (Sigma, St Louis, MO, USA). The mixture was mixed thoroughly and kept at -70℃ for long-term storage.

Plasmid DNA was extracted with the method described in 2.3.5.9. Restriction

enzyme digestion and automatic sequencing described in 2.3.5.10 and 2.3.5.11 were performed to confirm the plasmid DNA contained the target gene. The plasmid was linearized according to the method described above except Pvu II, which had one cutting site upstream of the T7 promoter region and another cutting site on the downstream of T3 promoter region, was used. The DIG-labeled RNA probe was produced and tested with the method described in 2.3.5.14.

2.3.7 Probe production for GAPDH

The GAPDH sequence was amplified using Taq DNA polymerase (Gibcol BRL, Grand Island, NY, USA) from the cDNA template. Forward primer (5'- acc aca gtc cat gcc atc ac – 3') and reverse primer (5' - tcc acc acc ctg ttg ctg ta – 3') (Life Technology, USA) were designed (size: 452bp) based on the sequence published in GeneBank database (assession no.: X02231). PCR reactions were performed in Peltier Thermal Cycler PTC200 (MJ Research Inc., Waltham, Massachusetts, USA) for 25 cycles and the condition was 94℃ for 1 minutes, 60℃ for 1 minutes and 72℃ for 2 minutes. After completing 25 cycles, the reaction mixture was heated at 72℃ for 10 minutes and was kept at 4℃ until use. The PCR product was separated and purified according the method described above.

DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Mannheim, Germany) was used for the production of DIG-labeled DNA probe. DIG-DNA labeling reaction was performed according to the manufacturer's protocol. Briefly, 1μg of GAPDH template DNA was added to a sterile, RNase-free microfuge tube and the final volume was filled up to 16μl by addition of autoclaved, double distilled water. The reaction mixture was heated to 95℃ for 10 minutes and chilled in ice bath quickly to denature the DNA template. The labeling of the DNA template was carried out by addition of DIG-High Prime (Roche, Mannheim, Germany) and incubated at

37℃ for 1 hour. After incubation, the reaction was stopped by heating the mixture to 65℃ for 10 minutes. The probe was stored at -70℃ until further use. The probe was tested with the method described in 2.3.5.14.

2.3.8 Northern blot

Total RNA was extracted according to the method described above. RNA samples were applied (25μg/lane) to a 1.5% agarose gel containing 0.41M formaldehyde and fractionated by horizontal gel electrophoresis at 55V for 4 hours. After electrophoretic fractionation, RNA was transferred overnight to a positively charged nylon membrane by capillary blotting and the membrane was fixed with UV crosslinker (Strategene, La Jolla, CA, USA).

Pre-hybridization was performed at 50 ℃ for 45 minutes in 25ml of pre-hybridization solution (DIG Easy Hub, Roche, Mannheim, Germany). Hybridization was at 50℃ overnight in 25ml of pre-hybridization solution containing oligonucleotide probe (100ng/ml). In the case of the probe specific for LDL-receptor gene, pre-hybridization and hybridization were set at 55 ℃. The following post-hybridization washes were performed: twice for 5 minutes in 2xSSC/0.1% SDS at room temperature, twice for 15 minutes in 0.1xSSC/0.1% SDS at 60℃ for LDL-receptor probe and 55℃ for HMG-CoA reductase/GAPDH probe.

The membranes were then rinsed briefly with washing buffer (0.1M maleic acid, 0.15M NaCl and 0.3% Tween-20, pH 7.5). They were blocked by incubation for 30 minutes at room temperature with blocking solution (Roche, Mannheim, Germany). After that, the membrane was incubated in 1x blocking solution with a polyclonal antibody against 1:10,000 digoxigenin (Fab fragment, Roche, Mannheim, Germany).

After being washed twice for 15 minutes with washing buffer, the membranes were rinsed for 5 minutes with detection buffer (0.1M Tris-HCl and 0.1M NaCl, pH

9.5) and immersed for 5 minutes in CDP-Star solution (Roche, Mannheim, Germany). Membranes were exposed to Fuji medical x-ray (SUPER RX) film for 15 to 60 minutes at room temperature.

Signals were scanned and quantified using the Sigma Scan software program (Jandel Scientific, San Rafael, CA, USA) and the level of mRNA for LDL-receptor and HMG-CoA reductase were estimated. The values were normalized to the corresponding amount of GAPDH mRNA.

2.3.9 Determination of hepatic cholesterol

Total lipids were extracted from 300mg of sample with addition of 1mg stimastanol as an internal standard, using 15ml chloroform-methanol (2:1, v/v) and 3 ml saline. The chloroform-methanol phase was removed after centrifugation and dried down under a gentle nitrogen steam. After an hour mild hydrolysis with 5ml NaOH in 90% ethanol at 90℃, 1ml of water and 6ml of cyclohexane were added for cholesterol extraction. The cyclohexane phase was removed after centrifugation and evaporated to dryness under a gentle nitrogen steam. Cholesterol extract was converted to their trimethyl-silyl (TMS)-ether derivatives by a commercial TMS reagent (dry pyridine-hexamethyldisilazane-trichlorosilane, 9:3:1 v/v/v, sil-A reagent; Sigma). After the incubation at 60℃ for an hour, the mixture was dried under nitrogen. The TMS-ether derivative was dissolved in 600μl of hexane, and after centrifugation, the hexane phase was transferred to a vial for gas-liquid chromatograph (GLC) analysis. Analysis of the cholesterol TMS-ether derivative was performed in a fused silica capillary column (SAC™-5, 30 m ´ 0.25mm, i.d.; Supelco, Inc., Bellefonte, PA, USA) in a Shimadzu GC-14B GLC equipped with a flame-ionization detector (Shimadzu, Tokyo, Japan). The column temperature was programmed at 285°C and maintained

for 20min. Helium was used as carrier gas at a head pressure of 22 psi. A typical profile of hepatic cholesterol and internal standard was showed in Figure 2.2.

2.3.10 Statistics

Results were presented as means±standard error of means (S.E.M.) of samples from 5 rats or hamsters. The statistical significance of differences between groups was assessed by one-way analysis of variance (ANOVA) followed by Student's *t*-test or Turkey's multiple comparison test, using Prism® (Graphpad software, Inc., CA, USA). Differences between groups were considered significant when $P<0.05$.

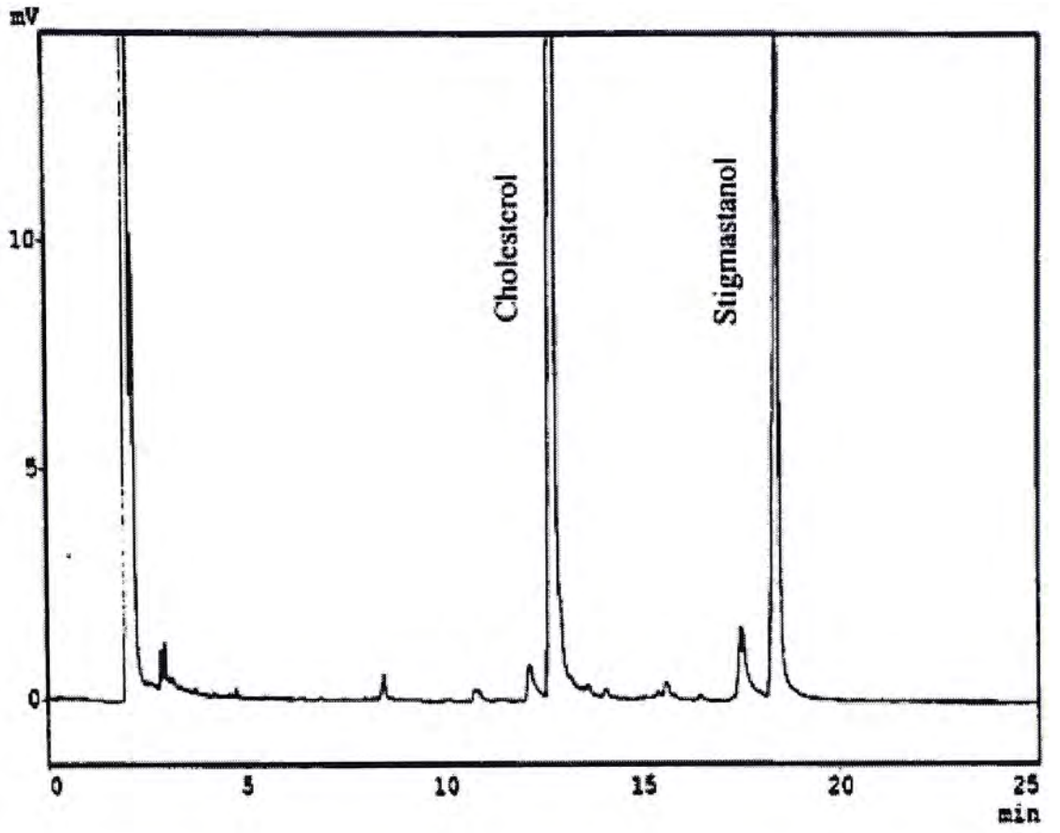

Figure 2.2. Gas liquid chromatographic profile of hepatic cholesterol and internal standard (stigmastanol).

2.4.1 Growth and food intake

Table 2.1 showed the changes in body weight and food intake of rats and hamsters after one month feeding, on various cholesterol diets. The initial and final body weight and daily food intake of rat in various groups showed no significant difference when compared with the control. Similarly, initial body weight and daily food intake of hamster in various groups showed no significant difference when compared with the control group. However, the final body weight of 0.3% and 0.9% groups showed a significant decrease when compared with the control group ($P<0.05$).

2.4.2 Effect of cholesterol supplements on serum cholesterol

Figure 2.1 showed the changes in serum total cholesterol (TC) in rats and hamsters maintained on various cholesterol diets for one month. The serum total cholesterol level of hamsters was much higher than that of the corresponding rat groups ($P<0.001$). The serum TC of rats increased to 142.4 ± 35.7mg/dl, in 0.9% diet group. In contrast, the serum TC level increased to 247.2 ± 29.7mg/dl in hamsters maintained on a 0.9% cholesterol diet.

Table 2.1. The changes in body weight and food intake of rats and hamsters maintained on the control diet (control, 0.0%), 0.3 % cholesterol diet (0.3%), 0.6 % cholesterol diet (0.6%) and 0.9% cholesterol diet (0.9%) for 1 month. Data are mean±S.E.M., n=5. * $P<0.05$.

	Experimental groups			
	Control	0.3%	0.6%	0.9%
Rats				
Initial body weight (grams/day/animal)	266.1 ± 9.3	252.2 ± 7.3	264.1 ± 2.4	266.3 ± 8.1
Final body weight (grams/day/animal)	474.0 ± 11.4	475.6 ± 8.5	469.2 ± 7.1	492.4 ± 10.8
Daily food intake (grams/day/animal)	30.7 ± 3.3	31.7 ± 3.8	31.0 ± 4.6	32.1 ± 3.8
Hamsters				
Initial body weight (grams/day/animal)	112.1 ± 1.2	113.2 ± 3.0	116 .7 ± 1.9	109.3 ± 4.0
Final body weight (grams/day/animal)	133.2 ± 3.7	118.6 ± 2.2	122.4 ± 2.6	118.6 ± 5.5
Daily food intake (grams/day/animal)	8.2 ± 1.3	8.5 ± 1.4	7.5 ± 1.5	7.9 ± 1.4

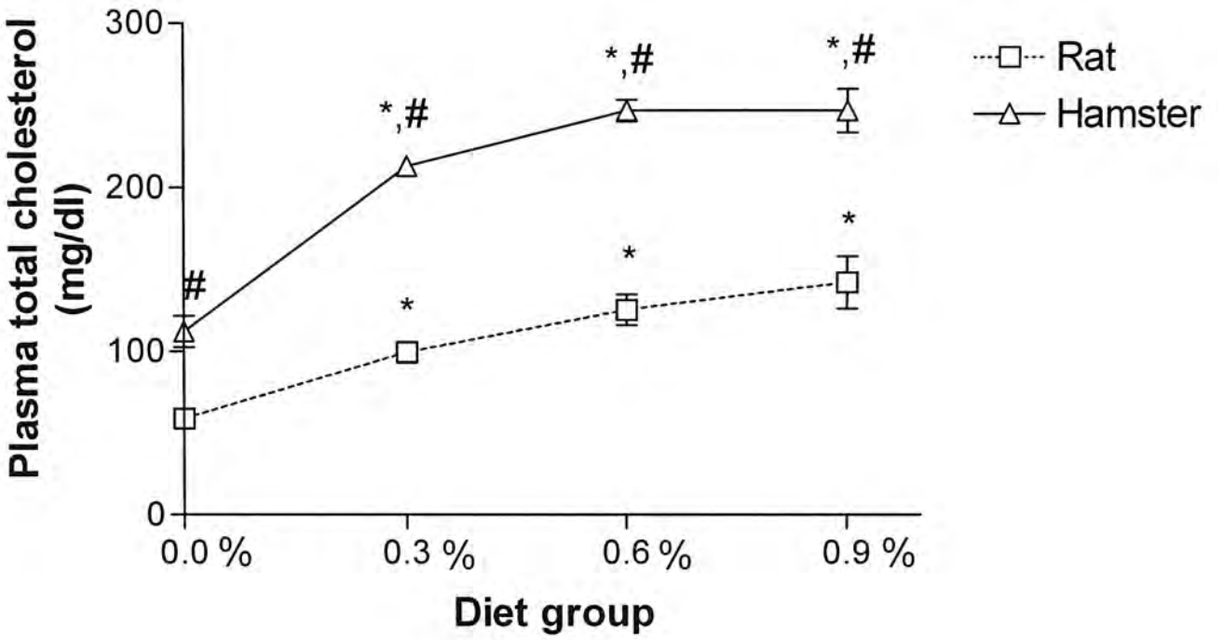

Figure 2.1. Changes in serum cholesterol level in rats and hamsters maintained on the control diet (0.0%), 0.3%, 0.6% and 0.9% cholesterol diet for one month. Data are means±S.E.M, n=5. * $P<0.05$ when compared with the control; # $P<0.001$ when compared with the rats at the same cholesterol level.

2.4.3 Effect of cholesterol supplements on liver cholesterol content

Figure 2.2 showed the liver cholesterol content of rat and hamster in various groups. The liver cholesterol content of rat had shown a stepwise increase from 7.6 ± 3.0mg/g to 23.5 ± 1.7mg/g with increasing level of chemical cholesterol supplement in diet. The liver cholesterol content of hamsters, interestingly, increased sharply from 5.5 ± 0.5mg/g for 0.0% group to 33.9 ± 3.9mg/g for 0.3% group. However, the liver cholesterol content of hamsters drop to 29.6 ± 5.7mg/g and 26.9 ± 1.9mg/g for the 0.6% and 0.9% group respectively.

2.4.4 Stimulatory effect of high cholesterol diet on nSREBP-2, LDL-receptor and HMG-CoA reductase in rats

Figure 2.3 showed immunoblots and group results of the nSREBP-2 in livers of rats feeding varying amount of cholesterol for one month. The amounts of the nSREBP-2 showed an increase in a concentration-dependent manner. Statistically significant increase was recorded for 0.6% and 0.9% cholesterol group where nSREBP-2 had increased by 115% ($P<0.05$) and 252% ($P<0.001$), respectively.

The gene of LDL-receptor was up-regulated by the transcription factor, nSREBP-2. Western blot analysis of LDL-receptor in membrane extracts and its group data were shown on Figure 2.4. The general expression pattern of the LDL-receptor was similar to that of nSREBP-2. Statistically significant increase was recorded for 0.6% and 0.9% cholesterol group where LDL-receptor had increased by 167% ($P<0.05$) and 274% ($P<0.001$), respectively.

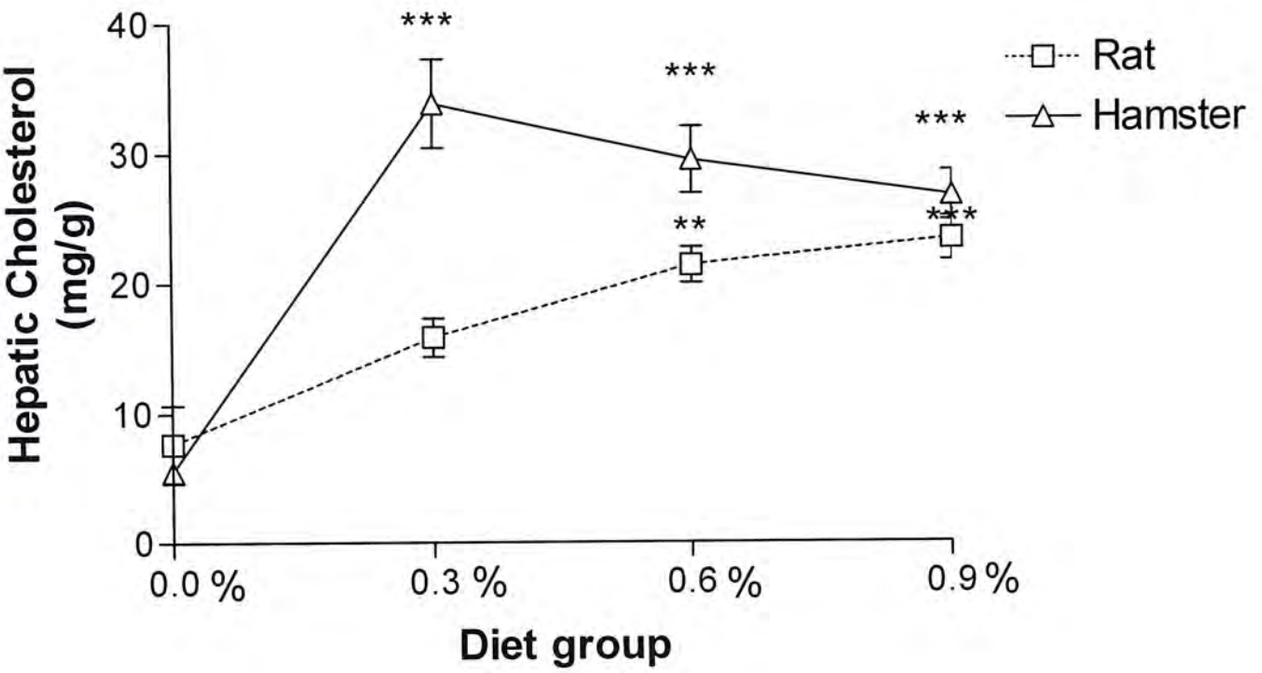

Figure 2.2. Changes in liver cholesterol level in rats and hamsters maintained on the control diet (0.0%), 0.3%, 0.6% and 0.9% cholesterol diet. Data are means±S.E.M., n=5. ** $P<0.01$ and *** $P<0.001$ when compared with the control.

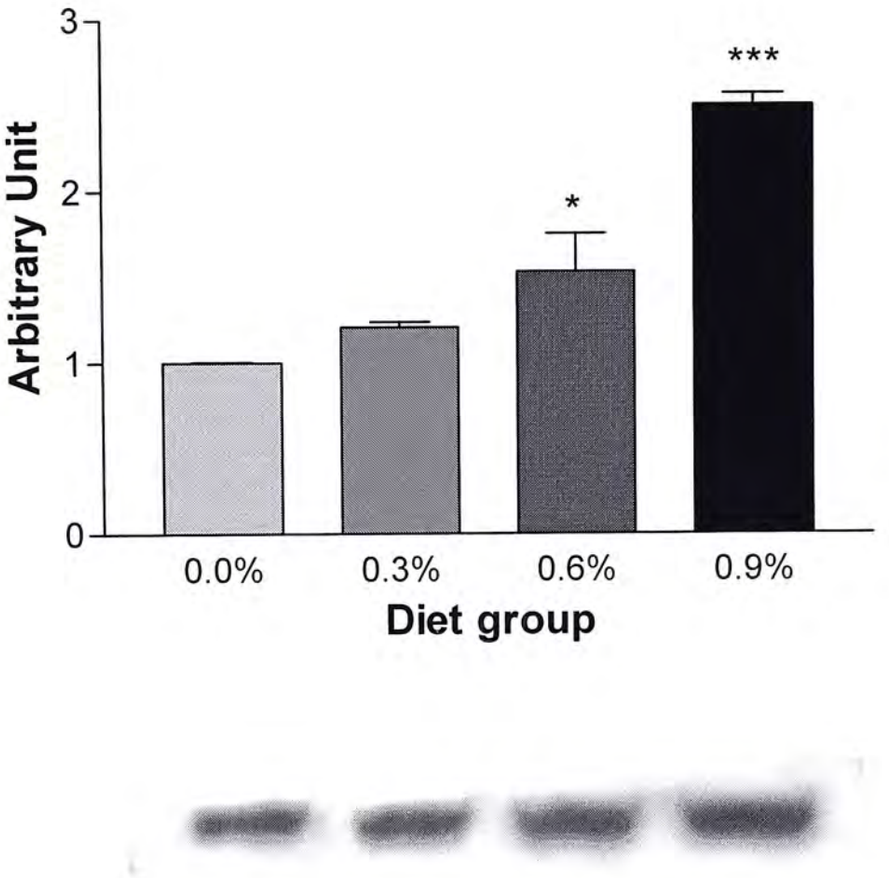

Figure 2.3. Representative Western blot and group data depicting nSREBP-2 abundance in the liver of Sprague-Dawley rats fed the control diet (0.0%), 0.3%, 0.6 and 0.9% cholesterol diet. Data are mean±S.E.M., n=5. * $P < 0.05$, *** $P<0.001$ when compared with the control.

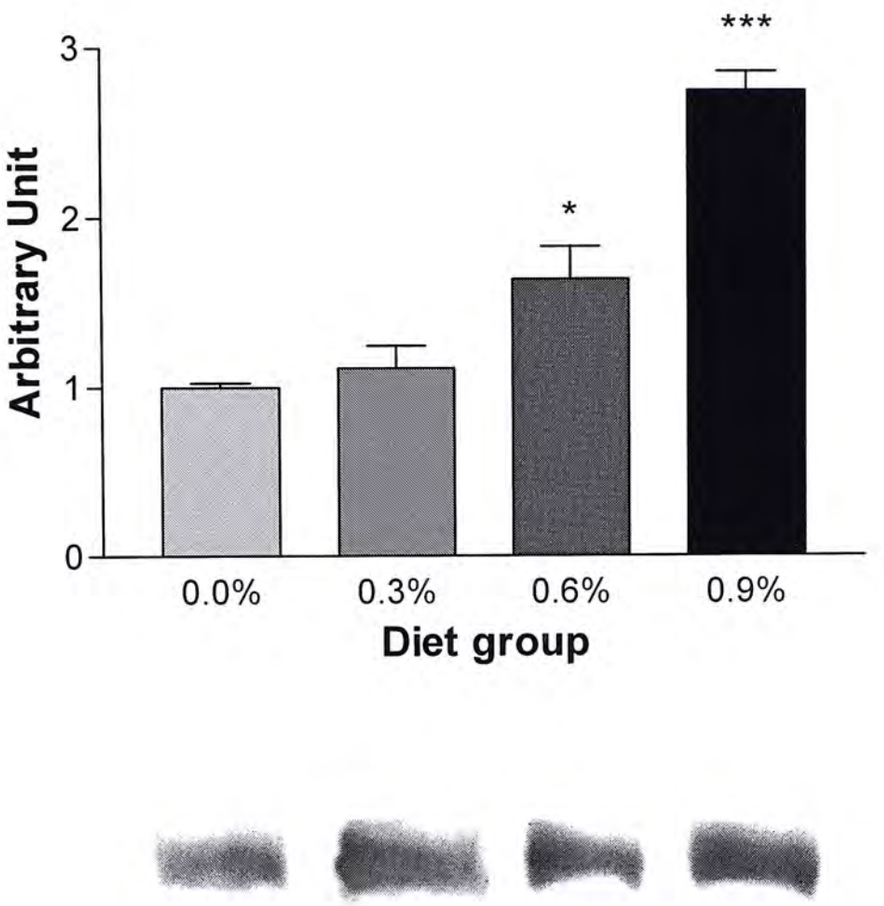

Figure 2.4. Representative Western blot and group data depicting LDL-receptor abundance in the liver of Sprague-Dawley rats fed the control diet (0.0%), 0.3%, 0.6 and 0.9% cholesterol diet. Data are mean±S.E.M., n=5. * $P < 0.05$, *** < 0.001 when compared with the control.

HMG-CoA reductase is another gene that can be up-regulated by nSREBP-2, which also acts as transcription factor. Western blot analysis of HMG-CoA reductase and its group data were shown in Figure 2.5. No change in the protein expression level of HMG-CoA reductase in rat liver was observed when dietary cholesterol was increased.

2.4.5 Effect of high cholesterol diet on nSREBP-2, LDL-receptor and HMG-CoA reductase in hamsters

Figure 2.6 showed immunoblots and group results of the nSREBP-2 in liver of hamsters fed various cholesterol diets. The amounts of nSREBP-2 showed no or little changes in response to dietary cholesterol when compared with the control diet. In fact, 0.3% to 0.9% diet groups had nSREBP-2 dropped slightly.

Western blot analysis of LDL-receptor and its group data are shown as Figure 2.7. It should be noted that while nSREBP-2, the transcription factor of LDL-receptor gene, showed slight decrease in its expression level in hamsters fed various high cholesterol diets. However, the LDL-receptor expression level did not drop according to the level of nSREBP-2. Changes in LDL-receptor expression among the groups were statistically insignificant when compared with the control.

Western blot analysis of HMGR and its group data are shown in Figure 2.8. No statistically significant change was observed in expression level of HMG-CoA reductase for different groups fed various cholesterol diets.

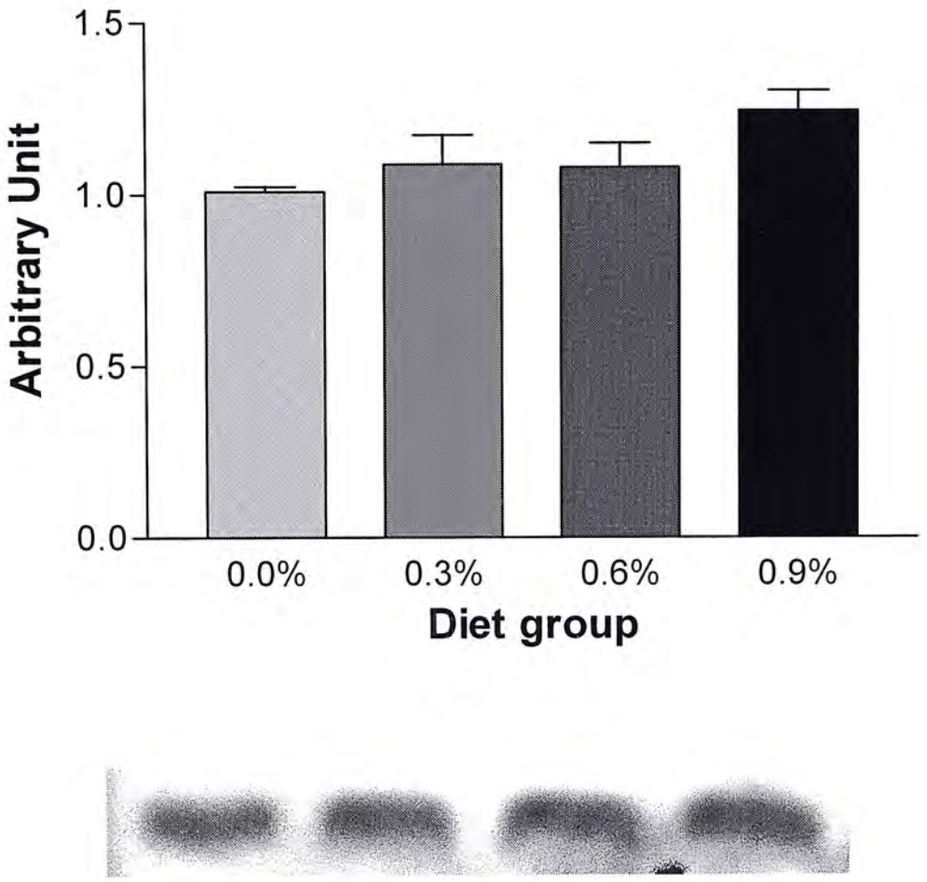

Figure 2.5. Representative Western blot and group data depicting HMG-CoA reductase abundance in the liver of Sprague-Dawley rats fed the control diet (0.0%), 0.3%, 0.6 and 0.9% cholesterol diet. Data are mean±S.E.M., n=5.

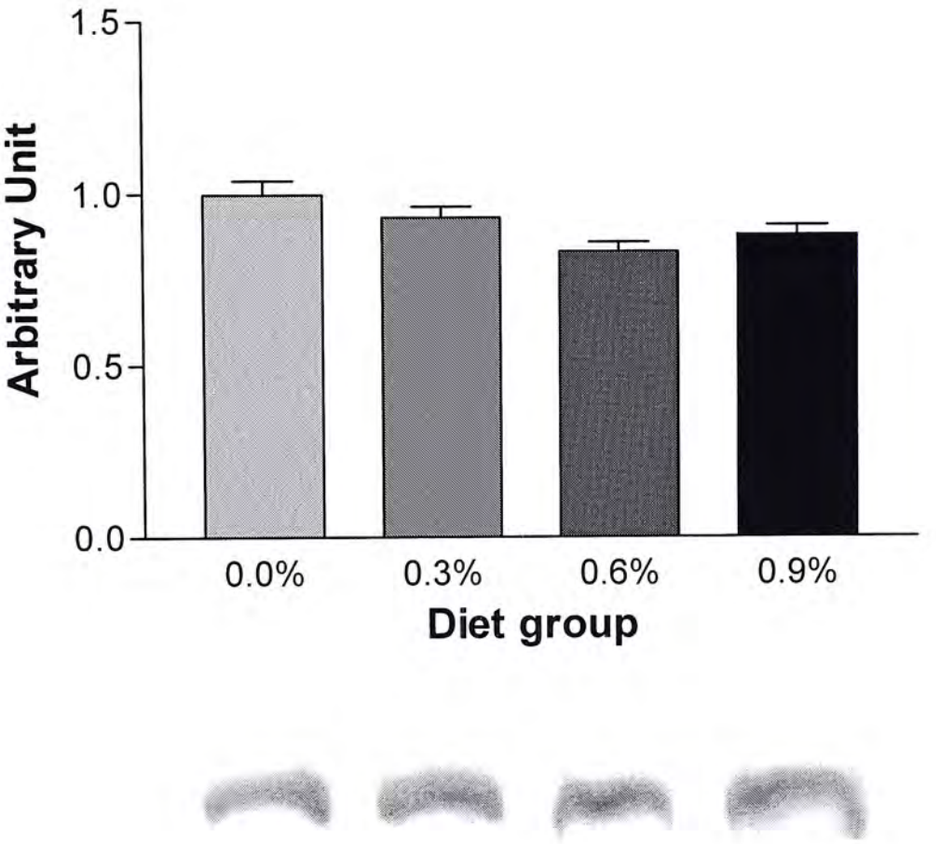

Figure 2.6. Representative Western blot and group data depicting nSREBP-2 abundance in the liver of Golden Syrian hamsters fed the control diet (0.0%), 0.3%, 0.6 and 0.9% cholesterol diet. Data are mean±S.E.M., n=5.

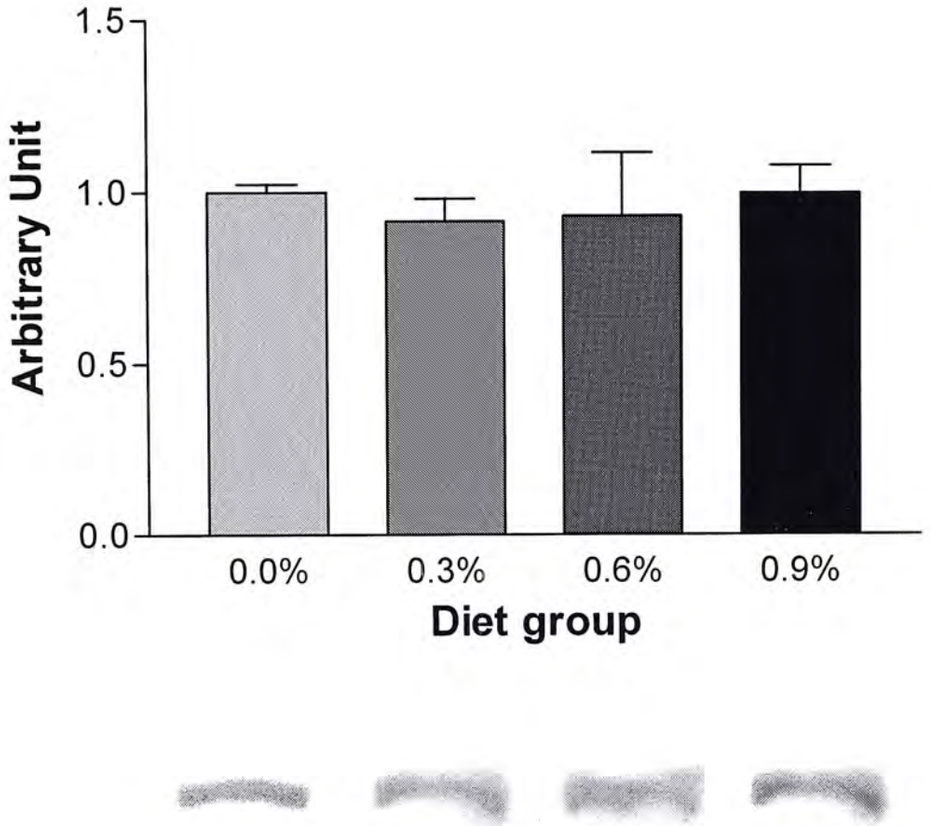

Figure 2.7. Representative Western blot and group data depicting LDL-receptor abundance in the liver of Golden Syrian hamsters fed the control diet (0.0%), 0.3%, 0.6 and 0.9% cholesterol diet. Data are mean±S.E.M., n=5.

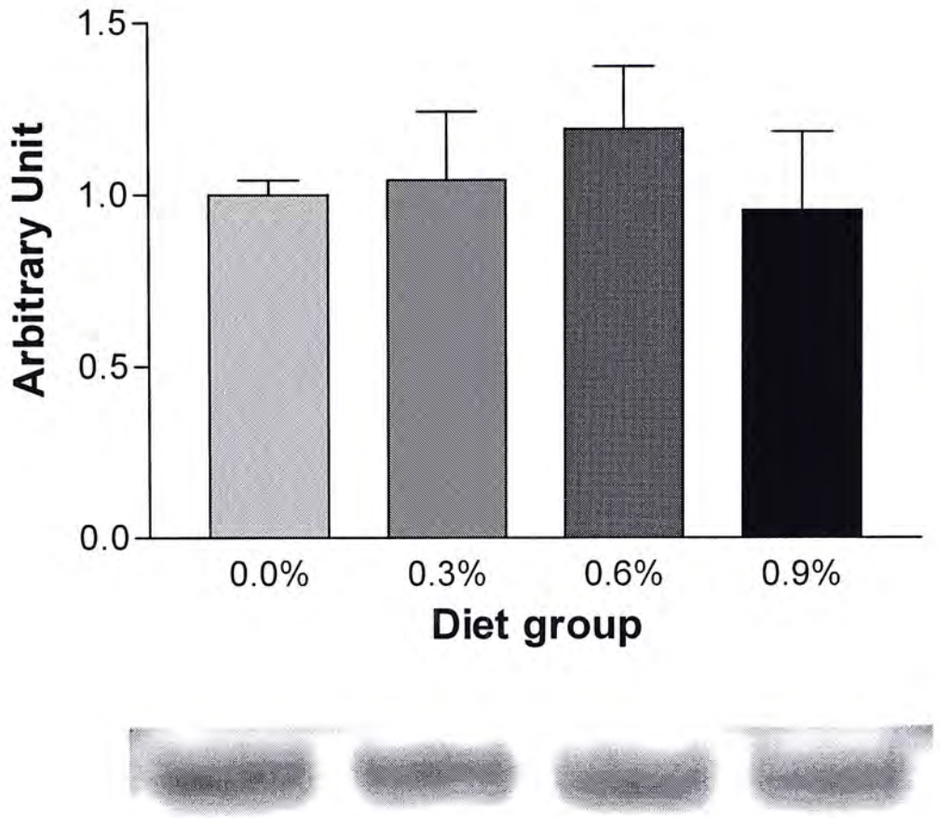

Figure 2.8. Representative Western blot and group data depicting HMG-CoA reductase abundance in the liver of Golden Syrian hamsters fed the control diet (0.0%), 0.3%, 0.6 and 0.9% cholesterol diet. Data are mean±S.E.M., n=5.

2.4.6 The regulation of LDL-receptor and HMG-CoA reductase existed at transcriptional level

The mRNA expression level of LDL-receptor and HMG-CoA reductase were measured in both rats and hamsters fed various cholesterol, using GAPDH to normalize the uneven loading of samples. In Figure 2.9, the mRNA expression level of LDL-receptor in rats showed a similar pattern as that observed in protein expression. Statistically significant increments were observed for 0.6% group (175 ± 20 %, $P < 0.05$) and 0.9% group (279 ± 10 %, $P < 0.05$), compared with the control. However, no statistically increment for HMG-CoA reductase mRNA expression, among all the rat groups was observed (Figure 2.10). For hamsters, no statistical significant change was observed in mRNA of LDL-receptor and HMG-CoA reductase, which was consistent with the protein expression pattern, (Figure 2.11 & 2.12).

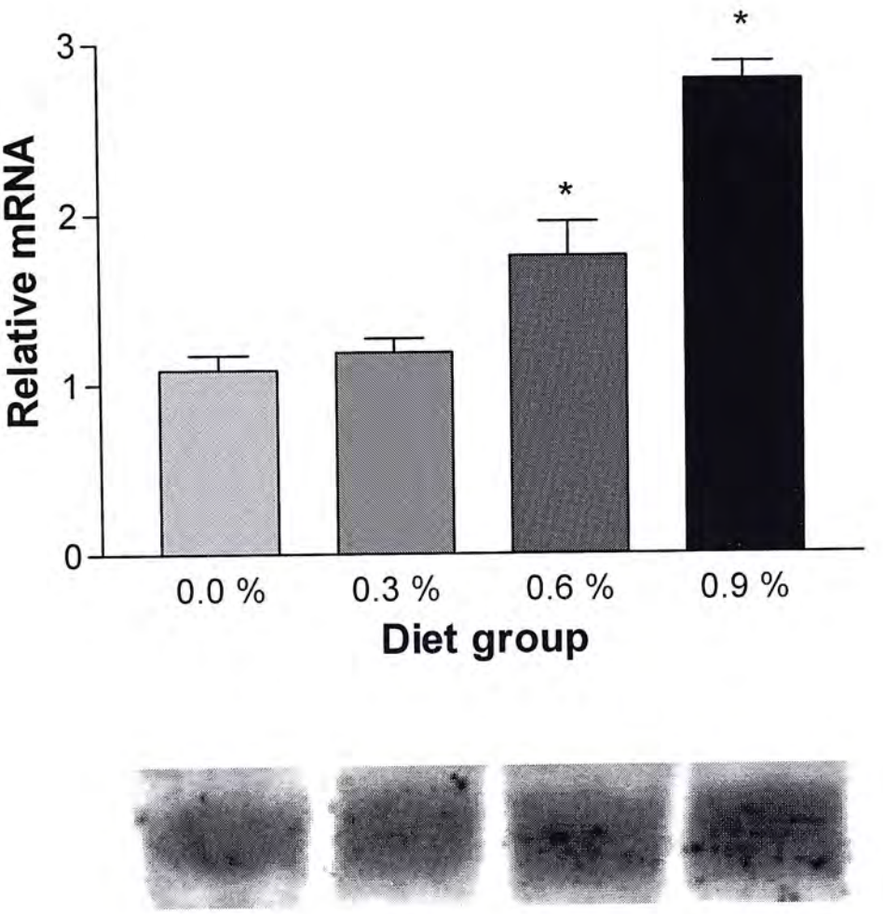

Figure 2.9. Representative Northern blot and group data depicting LDL-receptor mRNA abundance in the liver of Sprague-Dawley rats fed the control diet (0.0%), 0.3%, 0.6 and 0.9% cholesterol diet. Data are mean±S.E.M., n=5.* $P<0.05$ when compared with control.

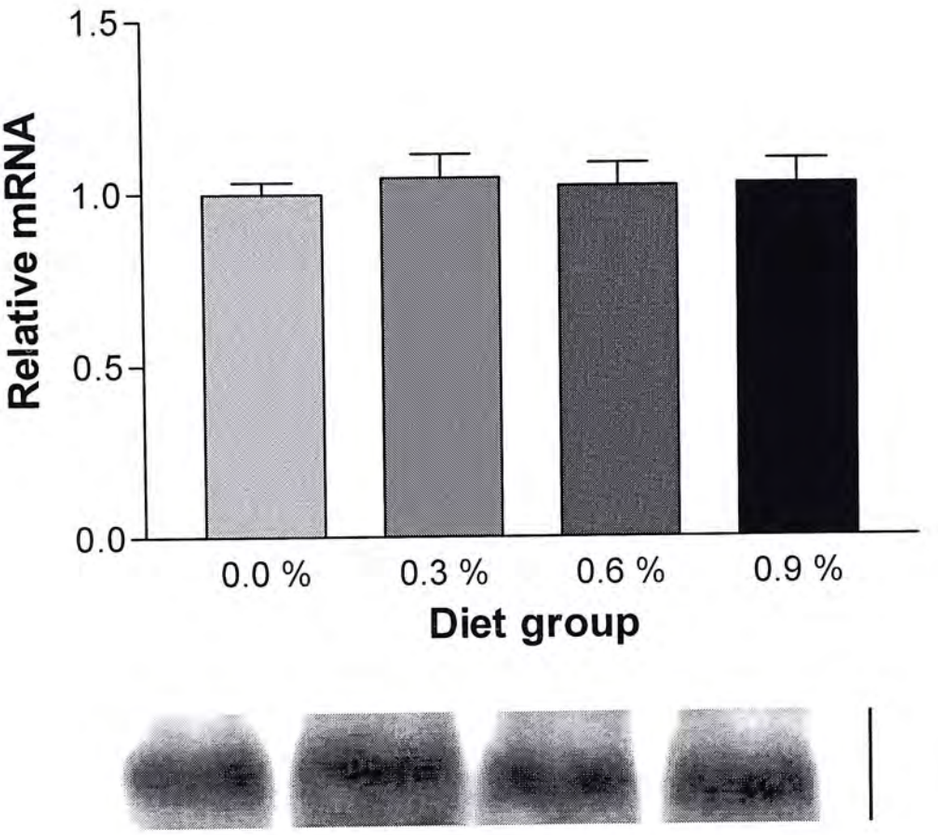

Figure 2.10. Representative Northern blot and group data depicting HMG-CoA reductase mRNA abundance in the liver of Sprague-Dawley rats fed the control diet (0.0%), 0.3%, 0.6 and 0.9% cholesterol diet. Data are mean±S.E.M., n=5.

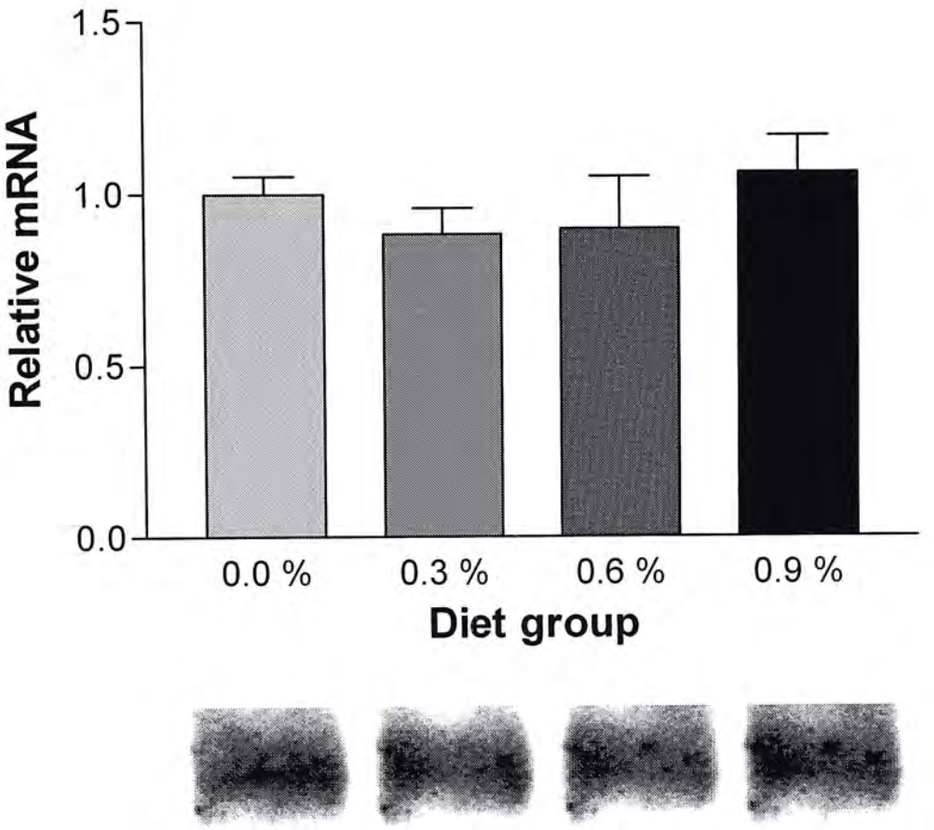

Figure 2.11. Representative Northern blot and group data depicting LDL-receptor mRNA abundance in the liver of Golden Syrian hamsters fed the control diet (0.0%), 0.3%, 0.6 and 0.9% cholesterol diet. Data are mean±S.E.M., n=5.

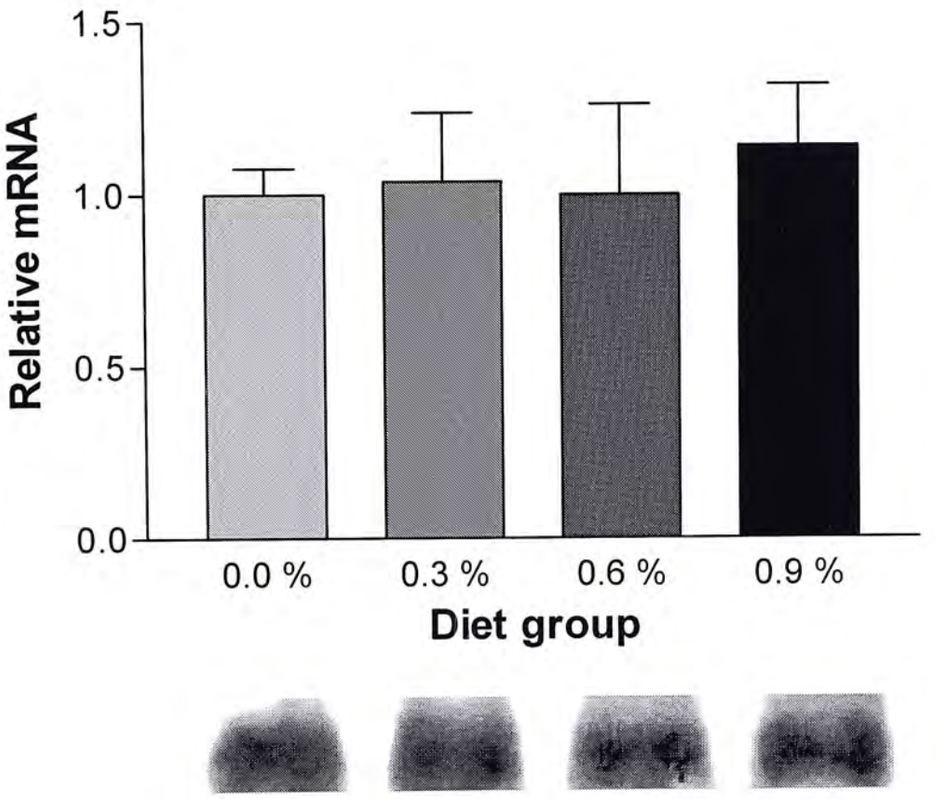

Figure 2.12. Representative Northern blot and group data depicting HMG-CoA reductase mRNA abundance in the liver of Golden Syrian hamsters fed the control diet (0.0%), 0.3%, 0.6 and 0.9% cholesterol diet. Data are mean±S.E.M., n=5.

2.5 Discussion

The present study clearly demonstrated that rats and hamsters responded differently to the dietary cholesterol. Rats did not necessarily have a sharp increase in serum cholesterol level in response to a higher cholesterol diet and were regarded as a "hypo-responsive" model. In contrast, hamster had a sharp increase in serum cholesterol level in response to increasing dietary cholesterol and thus was regarded as a "hyper-responsive" model in this experiment.

The present results showed that LDL-receptor in rats was up-regulated while that in hamsters was insensitive to dietary cholesterol. Up-regulation of LDL-receptor in rats would lead to efficient uptake of LDL-C by the liver and hence the level of serum cholesterol had not risen sharply in response to a high cholesterol diet. In this regard, the cholesterol content in rat liver had demonstrated a consistent result. With the increasing cholesterol levels in diet, the more liver cholesterol was detected. The present result was in agreement with that of Roach et al. (1993), who demonstrated that dietary cholesterol increased the mass of hepatic LDL-receptor in young rats. However, the LDL-receptor was not increased in response to dietary cholesterol in old rats. Therefore, it was clear that age may be a factor influencing the expression of LDL-receptor. In our experiment, the rats and hamsters used were of the similar age, so that their difference in LDL-receptor expression was not due to their age. In another study, deliberate over-expression of LDL-receptors had been suggested to have anti-hypercholesterolemic effect on mice fed with a high cholesterol diet (Yokode et al., 1990). Hence, it was clear that up-regulation of LDL-receptor is one of the mechanisms to prevent sharp increase of plasma cholesterol in rats.

Expression of HMG-CoA reductase is regulated mainly by a cellular cholesterol level. In response to a high cholesterol level in tissue, the expression level of

HMG-CoA reductase would decrease in order to maintain a constant level of cholesterol (Anderson et al., 1982). It had been suggested that the basal level expression of HMG-CoA reductase could be an indicator of tolerance to dietary cholesterol (Ness and Gertz, 2004a; 2004b; Ness and Chambers, 2000). The higher the basal expression of hepatic HMG-CoA reductase, the greater the "cholesterol buffering capacity" and the greater the resistance to dietary cholesterol. Interestingly, the rats have a high level of hepatic cholesterol biosynthesis and are known to be resistant to dietary cholesterol (Ness and Gertz, 2004b; Ness and Chambers, 2000; Spady and Dietschy, 1983). In contrast, hamsters exhibit a low level of HMG-CoA reductase and are very sensitive to dietary cholesterol (Spady et al., 1983). The present study did not found significant changes in hepatic HMG-CoA reductase of both rats and hamsters with the increasing cholesterol in the diet. Perhaps, 0.3% dietary cholesterol had repressed the expression of HMG-CoA reductase to its basal level. Further increment in dietary cholesterol (0.6% - 0.9%) could not further decrease the protein expression of HMG-CoA reductase. However, it should be noticed that rats have a greater basal expression of HMG-CoA reductase than hamsters. Even when both rats and hamsters had dropped their expression of HMG-CoA reductase to minimum level, the absolute reduction in cholesterol biosynthesis was actually greater in rats than hamsters (Spady et al., 1986). This was another mechanism accounted for the hypo-responsiveness of rats and hyper-responsiveness of hamsters to dietary cholesterol.

The expression of LDL-receptor is controlled by nSREBP-2. We further investigated whether the different response in two animals were due to the expression of nSREBP-2 in rats and hamsters. The gene expression of LDL-receptor in both animals was consistent with that of nSREBP-2. In rats, the higher expression level of nSREBP-2 was associated with the higher level of LDL-receptor gene expression. In

hamsters, the unchanged expression level of nSREBP-2 in response to the increasing dietary cholesterol was linked with unchanged LDL-receptor gene expression. This observation was in agreement with the previous reports showed parallel expression of nSREBP-2 with LDL-receptor mRNA (Makar et al., 1998; Mullen et al., 2004).

Although both LDL-receptor and HMG-CoA reductase contained sterol regulatory element (SRE-1) which could be activated by nSREBP-2, the gene expression profile of LDL-receptor and HMG-CoA reductase was different in rats. The gene expression of HMG-CoA reductase was not up-regulated in response to increase in nSREBP-2. It was suggested that the difference may be due to higher similarity of LDL-receptor promoter sequence than that of HMG-CoA reductase with SREBP-2 (Vallett et al., 1996). In addition, activation of HMG-CoA reductase transcription may require coupling with other factors, such as nuclear factor 1 (NF-1) (Osborne, 1991), which was not up-regulated by dietary cholesterol. Hence, the cell could turn off the cholesterol synthesis by lowing HMG-CoA reductase expression but maintaining high level of LDL-receptor to allow efficient utilization of plasma cholesterol (Osborne, 1991). This was consistent with the current study that the LDL-receptor was up-regulated while HMG-CoA reductase was not changed in response to increase in nSREBP-2. This regulatory mechanism was important for rats to prevent sharp increase in plasma cholesterol in response to dietary cholesterol.

The fundamental issue remains why nSREBP-2 was up-regulated in rats. It was demonstrated that the active (nuclear) form of SREBP-2, nSREBP-2, was produced from its precursor form (pSREBP-2) in response to the changing cholesterol level in the cell (Sakai et al., 1996). Low level of cellular cholesterol induces activation of SREBP-2, which up-regulates the expression of LDL-receptor gene, by binding to the SRE-1 region of the gene. On the other hand, when cellular cholesterol was high, SREBP-2 was deactivated and the expression of LDL-receptor was repressed (Hua et

al., 1993). However, the presnt results seemed to be contradictory to the above information. Despite the increasing dietary cholesterol had caused increase in hepatic cholesterol in rats, it still expressed higher level of nSREBP-2 to induce the expression of LDL-receptor at the transcriptional level.

In the body, cholesterol homeostasis is controlled in the liver mainly by three factors: (i) uptake from blood (by LDL-receptor), (ii) biosynthesis (by HMG-CoA reductase) and (iii) elimination outside the body as bile acids (by CYP7A1). It is hypothesized that the up-regulation of nSREBP-2 in rats but not in hamsters due to its greater inhibition of biosynthesis and more efficient elimination of hepatic cholesterol. First, rats had a higher rate of cholesterol synthesis than hamsters. For the same extent of decrease of HMG-CoA reductase, the absolute reduction of cholesterol biosynthesis was greater in rats than hamsters (Spady et al., 1986). Second, rats may have a greater ability to eliminate cholesterol from the liver than hamsters. Both the drop in biosynthesis and efficient elimination of cholesterol in rats has created a net cholesterol efflux across the liver (Figure 2.13). In order to compensate for the drop in hepatic cholesterol, more SREBP-2 was converted from its precursor form to the active nuclear form. This up-regulated the transcription of LDL-receptor and promoted the uptake of plasma cholesterol from blood, as observed in the present results. In contrast, hamsters may not be efficient in the elimination of hepatic cholesterol. Cholesterol content in the liver remained stable and the SREBP-2 did not sense a need to up-regulate the nSREBP-2. As a result, the LDL-receptor was not up-regulated in hamsters with increasing dietary cholesterol. This hypothesis was supported by the results in chapter 3. In rats, the higher level of dietary cholesterol had stimulated a higher expression of CYP7A1 and excreted more fecal bile acids, indicating more efficient elimination of cholesterol. Conversely, hamsters are less efficient in cholesterol elimination as shown by unchanged CYP7A1 protein

expression and fecal bile acids in response to a higher dietary cholesterol.

To summarize, rats were hypo-responsive to dietary cholesterol because they expressed higher level of LDL-receptor with the expression of HMG-CoA reductase being unchanged (Figure 2.14). This process was regulated at transcriptional level that was controlled by the active form of the transcription factor, SREBP-2. In contrast, hamsters were hyper-responsive to dietary cholesterol because they lack of response in LDL-receptor expression to high cholesterol diets (Figure 2.15).

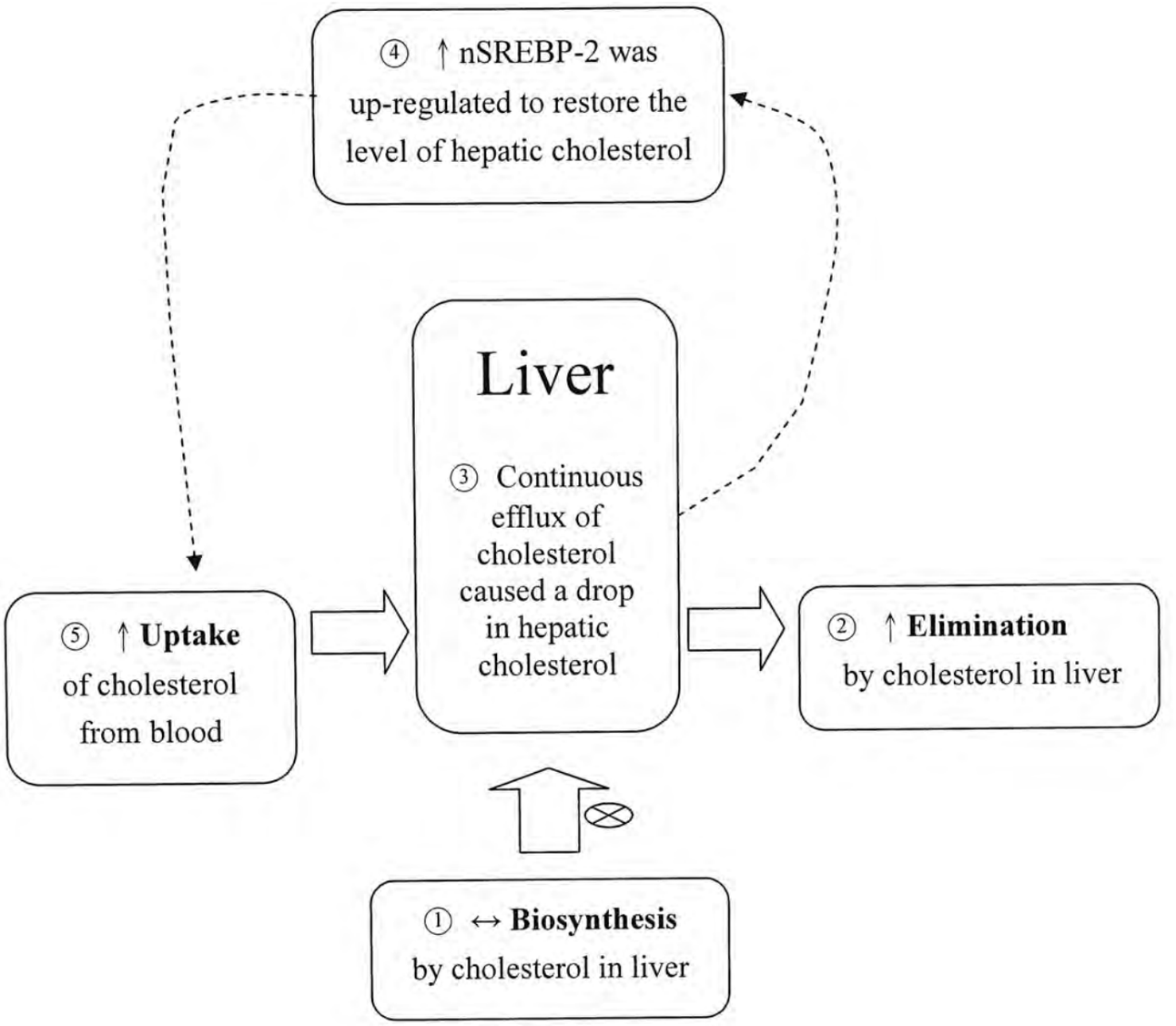

Figure 2.13. Schematic diagram to show the mechanism for the up-regulation of SREBP-2 in rats in response to high dietary cholesterol.

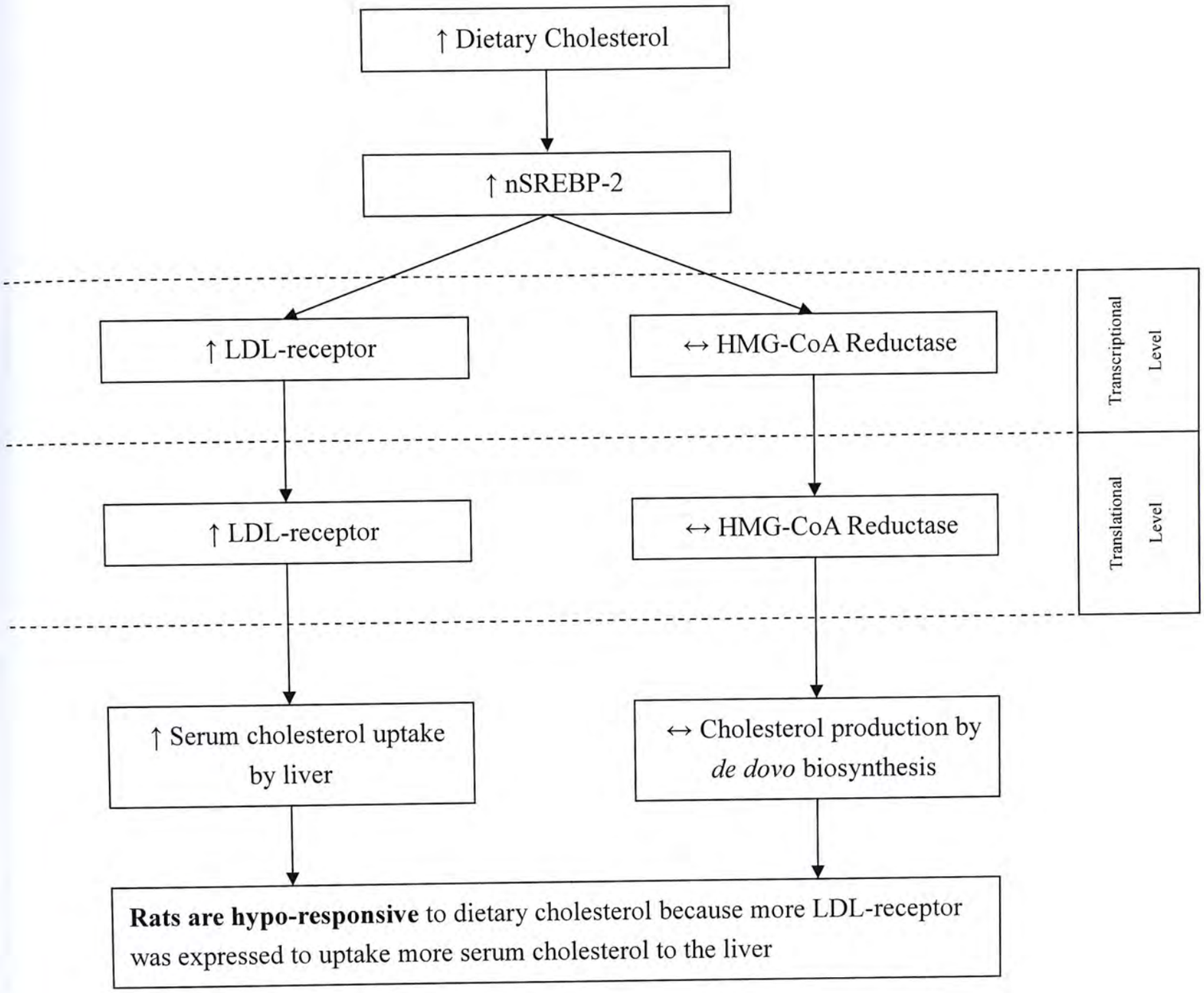

Figure 2.14. Illustration of the hypo-responsive mechanism of rats in response to a high dietary cholesterol.

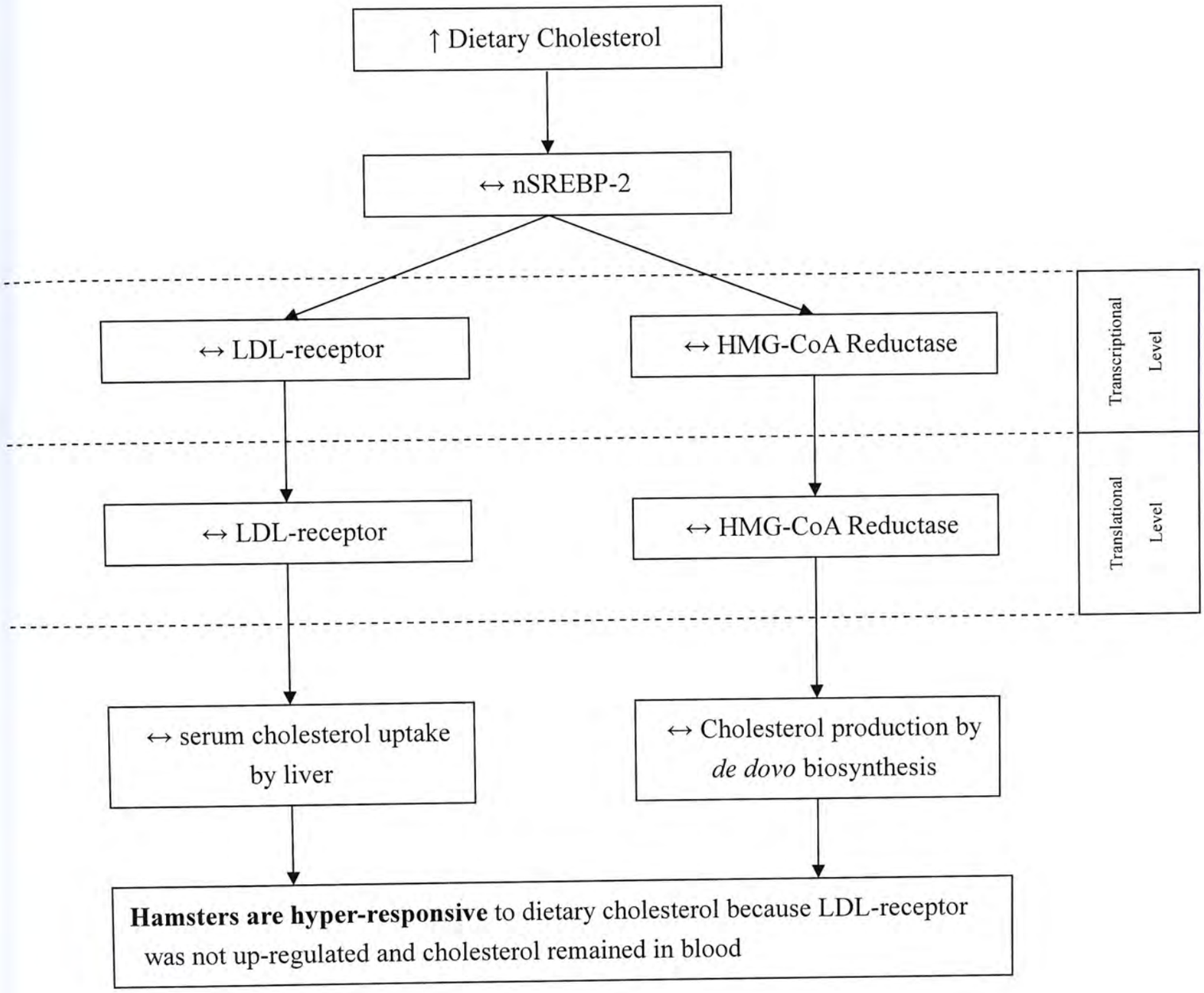

Figure 2.15. Illustration of the hyper-responsive mechanism of hamsters in response to a high dietary cholesterol.

CHAPTER 3

Rats are hypo-responsive to dietary cholesterol due to efficient elimination of cholesterol

3.1 Introduction

In the body, the cholesterol elimination is mainly controlled by the liver, where excess cholesterol is converted to bile acids before they are eliminated. Figure 3.1 illustrated the pathway of cholesterol elimination from cholesterol into bile acids. The majority of bile acids are biosynthesized by the classical pathway, which is controlled by a liver-specific rate-determining enzyme, CYP7A1 (Russell et al., 1992). It was found that up-regulation of LXR-α is associated with enhanced CYP7A1 expression in some animals, indicating LXR-α is a positive regulator of CYP7A1 (Gupta et al., 2002; Chiang et al., 2001; Peet et al., 1998a). In turn, the activation of LXR-α is controlled by a substrate-mediated pathway, which requires binding of cholesterol (oxysterol) as ligands (Forman et al., 1997; Janowski et al., 1996). The activated LXR-α forms a heterodimer with retinoid X receptor (RXRs), which acts as a transcription factor binding to the LXR responsive element (LXRE) of CYP7A1 gene and up-regulates its transcription (Willey et al., 1997).

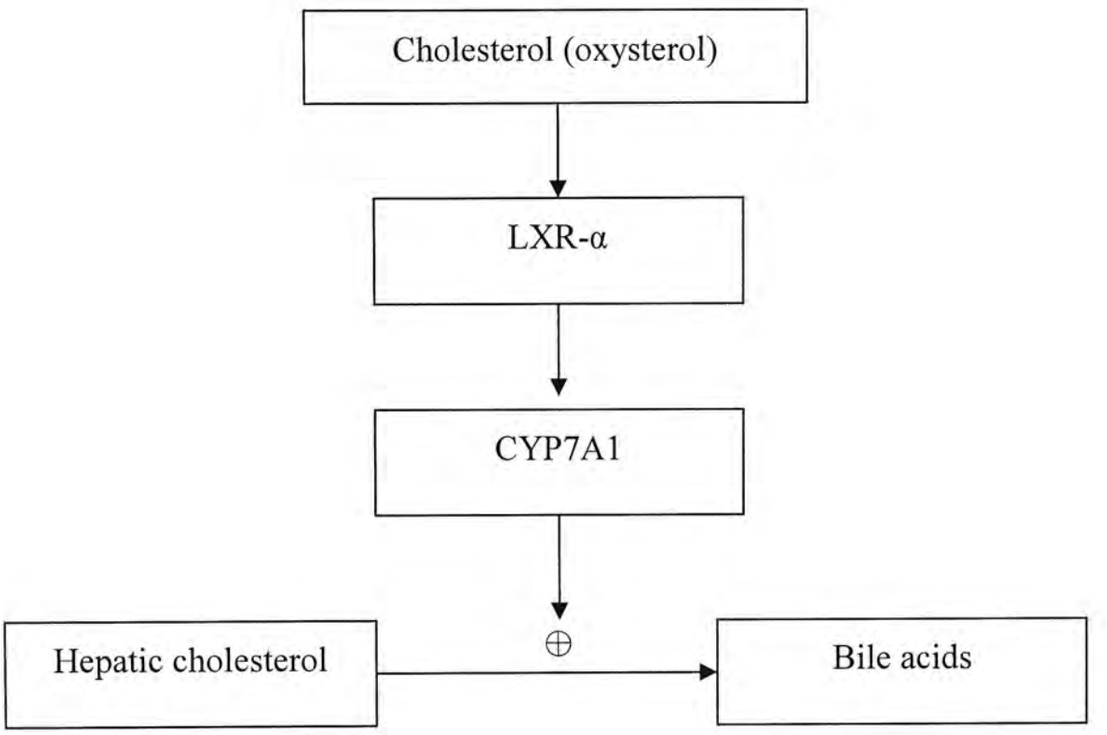

Figure 3.1. Illustration of the classical pathway involved in the conversion of cholesterol to bile acids.

3.2 Objective

Cholesterol homeostasis is a balance of absorption and elimination. It was previously demonstrated that rats were hypo-responsive whereas hamsters were hyper-responsive to dietary cholesterol. This study was extended from chapter 2 to answer the question, whether the up-regulation of nSREBP-2 and LDL-receptor in rats was due to more efficient elimination of cholesterol than hamsters. In addition, the mechanism responsible for the different cholesterol elimination efficiency, if any, was determined.

3.3 Methods and materials

3.3.1 Animals and diets

Both Sprague-Dawley (SD) rats and Golden Syrian (GS) hamsters were divided into four groups (n=5 each group) and were maintained on one of four diets: 0.0%, 0.3%, 0.6% and 0.9% cholesterol. The feeding conditions were previously described in Chapter 2.

3.3.2 Western blot

The total liver proteins and membrane proteins were obtained as previously described in Chapter 2. For the measurement of CYP7A1, the membrane protein (100μg) was size-fractionated on 8% SDS-PAGE at 120V for two hours. After electrophoresis, proteins were transferred to a Hybond-ECL membrane (Amersham Life Science Inc., Arlington Heights, IL, USA). The membrane was incubated for one hour in blocking solution (1x TBS, 0.1% Tween-20 and 5% nonfat milk) at room temperature and then overnight at 4℃ in the same solution containing 1:300 anti-CYP7A1 antibody (Santa Cruz Biotechnology, Inc., California, USA). Membrane was washed once for 15 minutes then twice for five minutes in washing solution (1x TBS and 0.1% Tween-20) prior to one hour incubation in blocking solution containing diluted (1:3000) horseradish peroxidase-linked rabbit anti-goat IgG (Zymed Laboratories Inc., South San Francisco, USA). The washes were repeated before the membranes were developed with chemiluminescent agents (ECL; Amersham Life Science) and subjected to autoradiography for one to five minutes.

For LXR-α, the total protein (50μg) aliquots were size-fractionated on 8% SDS-PAGE at 120V for two hours. The whole procedures were as above described except for 1:300 anti-LXR-α antibody (Santa Cruz Biotechnology, Inc., USA) and

1:3000 donkey anti-rabbit IgG (Amersham Life Science Inc., Arlington Heights, IL, USA) was used as primary and secondary antibody, respectively.

3.3.3 Probe production for CYP7A1 and LXR-α

The procedure for RNA probe production was similar to HMG-CoA reductase as previously described in 2.3.6 except the expressed sequence-tagged (EST) clone for CYP7A1 (Image ID: 1 782 989) and LXR-α (Image ID: 2 695 317) was purchased from ATCC (VA, USA).

3.3.4 Northern blot

The Northern blot was carried out as previously described in 2.3.8. Hybridization and post-hybridization temperature were set at 50℃ and 55℃, respectively. Signals were scanned and quantified using the Sigma Scan software program (Jandel Scientific, San Rafael, CA, USA) and the level of mRNA for CYP7A1 and LXR-α were estimated. The values were normalized to the corresponding amount of GAPDH mRNA.

3.3.5 Determination of fecal neutral and acidic sterols

The fecal neutral and acidic sterols were determined according to Czubayko et al. (1991) with some modifications.

3.3.5.1 Separation of neutral and acidic sterols

Fecal samples were first dried in a lyophilizer and then grinded. Stigmasterol (0.3mg/ml chloroform) and hyodeoxycholic acid (0.3mg/ml 1N NaOH in 90% ethanol) were added to 300mg grinded fecal sample as internal standards for neutral and acidic

sterols, respectively. The samples were then subjected for alkaline hydrolysis in 8ml 1N NaOH in 90% ethanol at 90℃ for 1 hour followed by cooling down to room temperature. After that, 1 ml of distilled water and 8 ml of cyclohexane were added for extraction. After centrifugation, the upper cyclohexane phase and the lower aqueous phase were collected for neutral and acidic sterols analyses, respectively.

3.3.5.2 Neutral sterols analysis

The cyclohexane phase from the above process was evaporated to dryness under a gentle nitrogen stream. The neutral sterols were then converted to their TMS-ether derivatives by adding TMS-reagent (Sil-A reagent, Sigma) and heated at 60℃ for 1 hour. The mixture was then dried under nitrogen stream again and the TMS-derivatives of neutral sterols were dissolved in 400μl of hexane. After centrifugation, the hexane layer was transferred to a vial for GLC analysis.

3.3.5.3 Acidic sterols analysis

For the lower aqueous phase extracted from the above process, 1ml of 10N NaOH was added to the lower aqueous phase and the mixture was then heated for 3 hours at 120℃. After adding 3ml distilled water and cooling to the room temperature, the mixture were acidified to pH < 1 with 25% HCl. The acidic sterols were extracted twice with 7ml diethyl ether. The combined ether phase were evaporated under nitrogen stream. Methylation was performed by adding 2ml methanol, 2ml dimethoxypropane, and 40μl concentrated HCl. The samples were mixed thoroughly and allowed stand at room temperature for overnight. The acidic sterols were then dried under nitrogen stream and converted to their TMS-ether derivatives as described for neutral sterols. The dried samples were dissolved in 300μl hexane and then centrifuged. The hexane phase was transferred to a vial for GLC analysis.

3.3.5.4 GLC analysis of neutral and acidic sterols

The GLC of fecal neutral and acidic sterols was carried out on a fused silica capillary column as described in 2.3.9. For neutral sterols, the column temperature was programmed at 285℃ and maintained for 30 minutes. For the acidic sterols, the column temperature was programmed at 230-280℃ at a rate of 1℃/min. Helium was used as the carrier gas at a head pressure of 22 psi in both neutral and acidic sterol GLC. Typical chromatogram of neutral and acidic sterols were shown in Figure 3.2 and 3.3, respectively.

3.3.6 Statistics

Results were presented as means±standard error of means (S.E.M.) of samples from 5 rats or hamsters. The statistical significance of differences between groups was assessed by one-way analysis of variance (ANOVA) followed by Student's *t*-test or Turkey's multiple comparison test, using Prism® (Graphpad software, Inc., CA, USA). Differences between groups were considered significant when $P<0.05$.

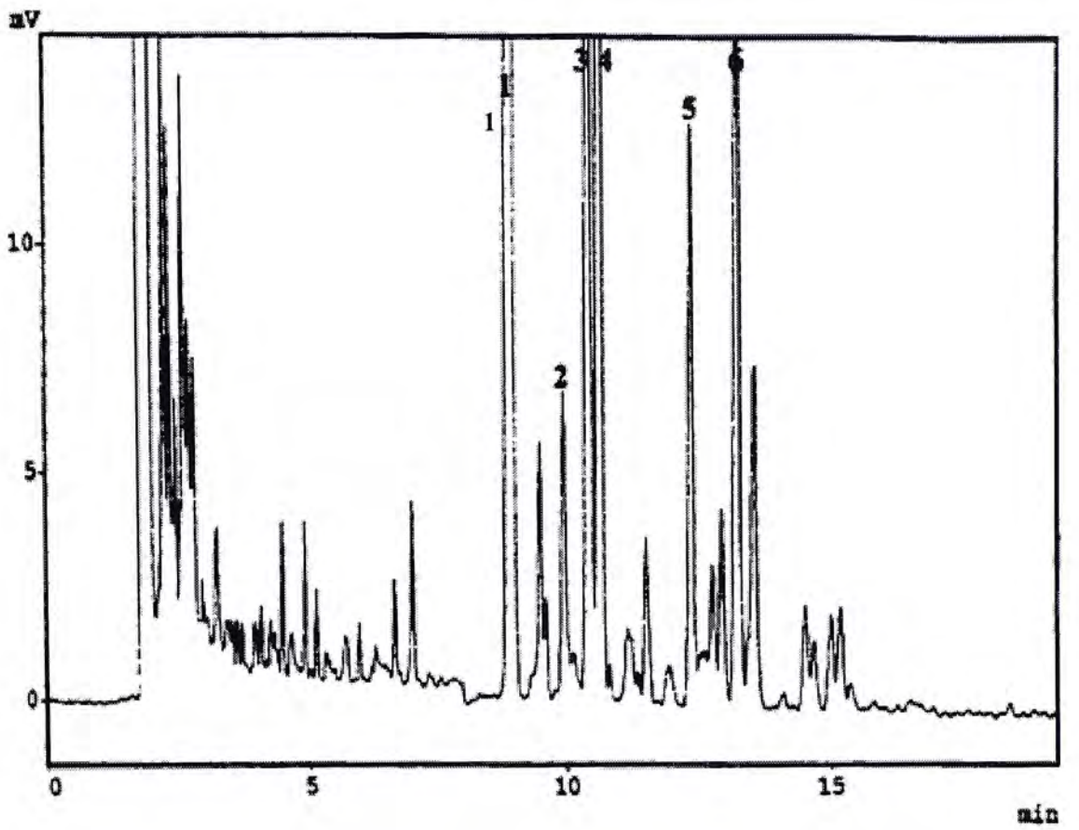

Figure 3.2. Typical gas liquid chromatographic profile of fecal neutral sterols. Identification of peaks: 1, coprostanol; 2, coprostanone; 3, cholesterol; 4, dihydrocholesterol; 5. campersterol; 6, internal standard (stigmasterol).

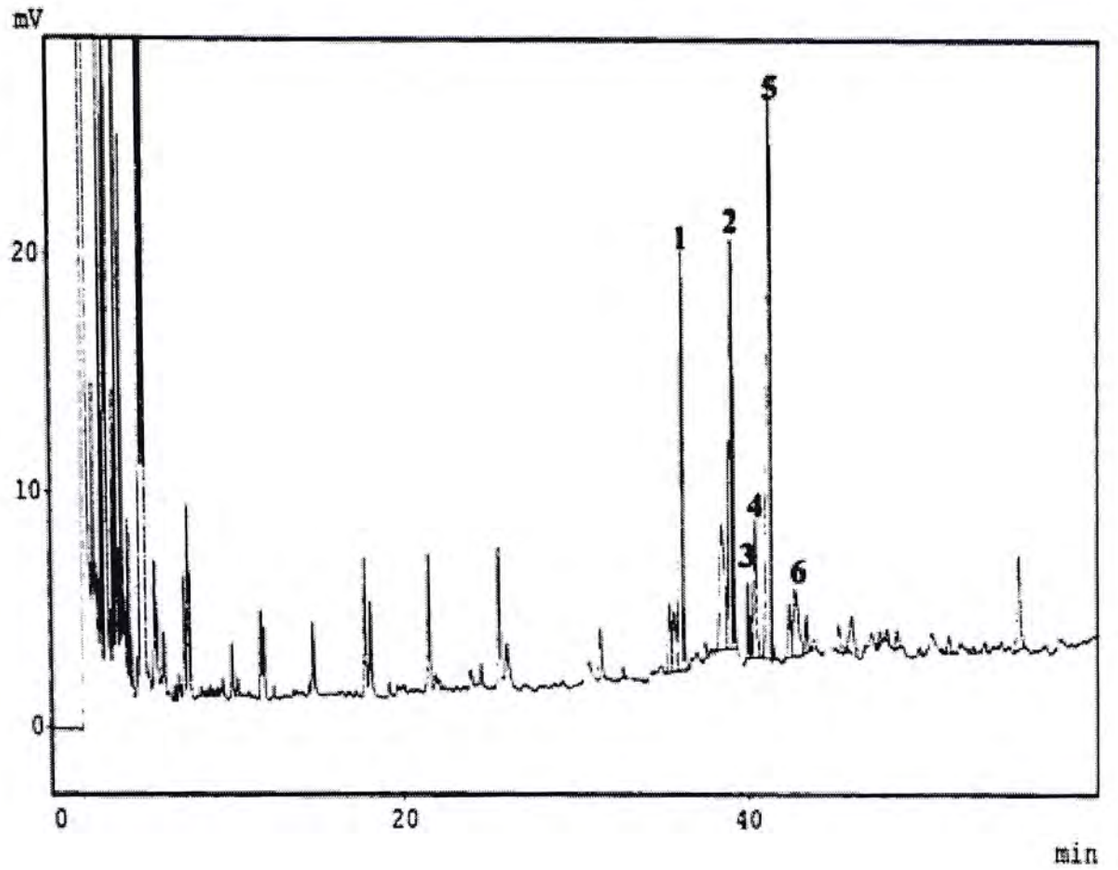

Figure 3.3. Typical gas liquid chromatographic profile of fecal acidic sterols. Identification of peaks: 1, lithocholic acid; 2, deoxycholic acid; 3, chenodeoxycholic acid; 4, cholic acid; 5, hyodeoxycholic acid (internal standard); 6, ursodeoxycholic acid.

3.4 Results

3.4.1 Effect of cholesterol supplements on fecal total neutral sterols

Fecal neutral sterols represent the unabsorbed cholesterol in the intestine. Figure 3.4 showed the fecal neutral sterol output by rats and hamsters from different diet groups. The fecal neutral sterols output by rats showed a sharp increase with the increasing of cholesterol content in the diet. The fecal neutral sterols output by rats constituted about 6.65 ± 0.13mg/g for the control group (0.0%), while it increased up to 29.66 ± 3.02mg/g ($P<0.05$), 54.32± 5.55mg/g ($P<0.01$) and 129.50 ± 0.82mg/g ($P<0.001$) for the 0.3%, 0.6% and 0.9% cholesterol groups, respectively. Although an increase across the diet groups with the increasing dietary cholesterol was also recorded in hamsters, the total fecal neutral sterols excretion was much less than that in the corresponding rat group.

3.4.2 Effect of cholesterol supplements on fecal total bile acids

Fecal total acidic sterols (bile acids) were measured to determine the amount of bile acids excreted in feces. Figure 3.5 showed the fecal bile acids output of rats and hamsters in different diet groups. The starting levels of fecal bile acids output for both animals were similar, which was about 0.6mg/g fecal bile acids for 0.0% cholesterol diet group. The fecal bile acids of rats increased to 2.09 ± 0.26mg/g for 0.3% cholesterol diet group. After that, the fecal bile acids output raised sharply to 33.46 ± 4.14mg/g for 0.6% cholesterol diet group and reached the maximum of 37.53 ± 4.64mg/g for 0.9% cholesterol diet group. In contrast, the fecal bile acids output in hamsters remained at the level of below 1 mg/g among all the diet groups.

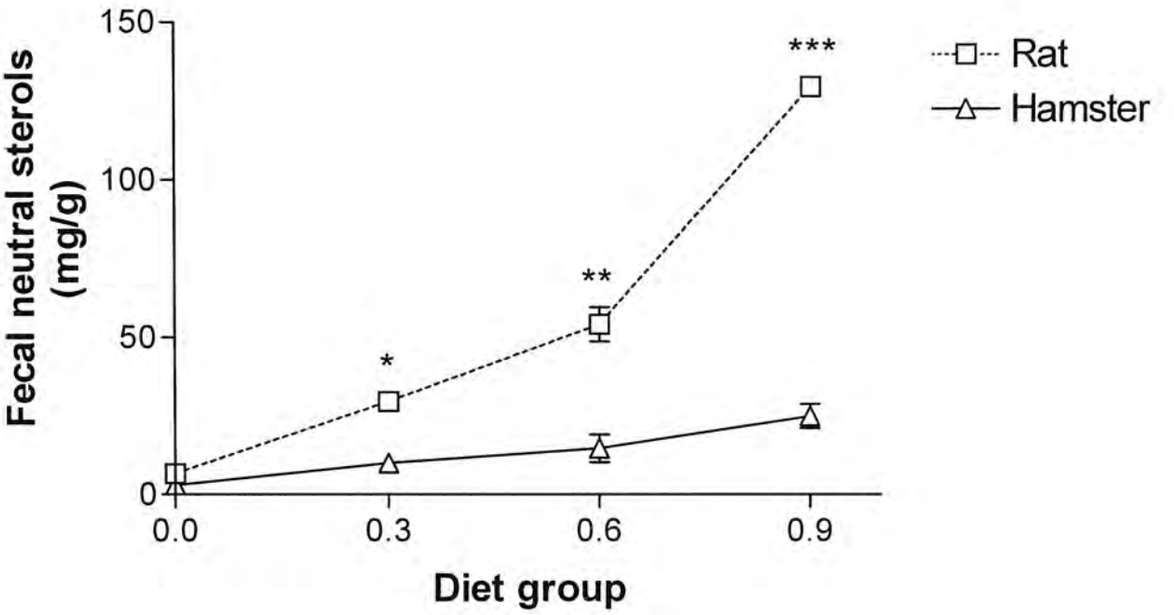

Figure 3.4. Fecal output of total neutral sterols in rats and hamsters maintained on the control diet (0.0%), 0.3% 0.6% and 0.9% cholesterol diet for one month. Data are means±S.E.M, n=5. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ when compared with the control.

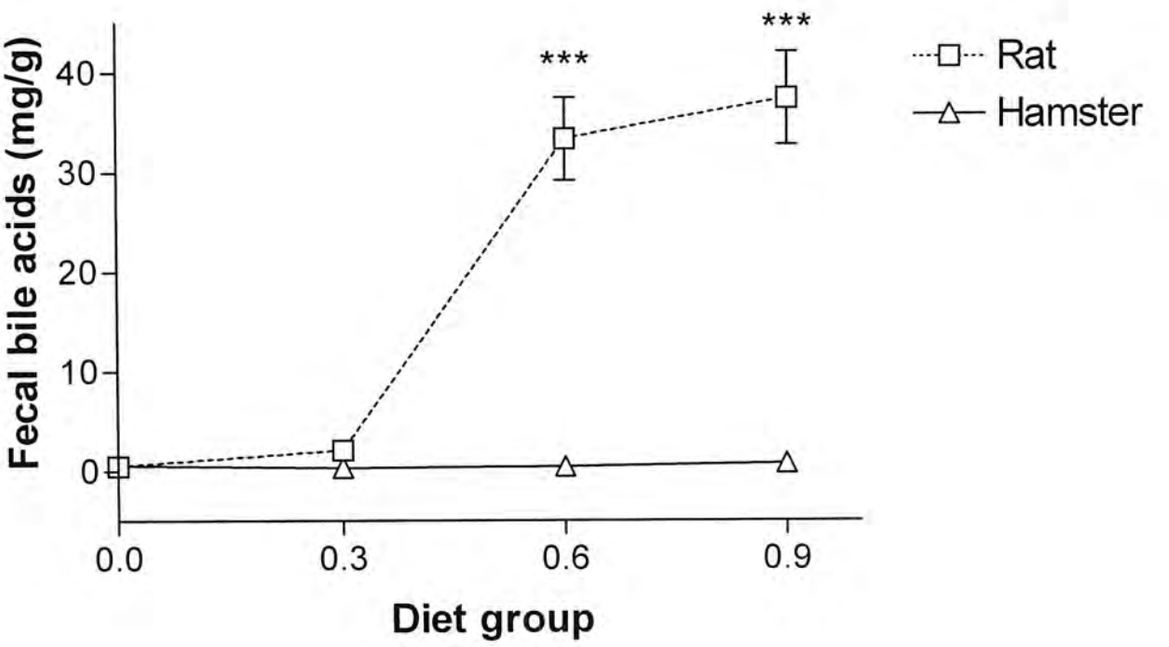

Figure 3.5. Fecal output of total bile acids in rats and hamsters maintained on the control diet (0.0%), 0.3% 0.6% and 0.9% cholesterol diet for one month. Data are means±S.E.M, n=5. *** $P<0.001$ when compared with the control.

3.4.3 CYP7A1 protein on rats showed a concentration-dependent increase with response to dietary cholesterol while hamsters did not

CYP7A1 catalyzes the rate-controlling reaction in the classic bile acid synthesis pathway. Figures 3.6 and 3.7 showed the immunoblots and group results of CYP7A1 protein in rats and hamsters fed various high cholesterol diets. CYP7A1 protein expression increased with the increasing dietary cholesterol. Statistically significant increase was found with the groups treated with 0.9 % diet (2.56 ± 0.45, $P < 0.05$). For hamster groups, however, no statistically significant change in CYP7A1 protein expression was observed among all diet groups.

3.4.4 The regulation of CYP7A1 was at transcriptional level

The mRNA expression level of CYP7A1 was also analyzed in both animals, using GAPDH to normalize the uneven loading of samples. Figure 3.8 showed the immunoblot and group results of the CYP7A1 mRNA expression in the liver of rats fed various cholesterol diets for a month, while Figure 3.9 for that of hamsters. It was observed that CYP7A1 mRNA expression of rats was up-regulated in accordance to the increasing amount of cholesterol in the diet. However, no significant change across the four diet groups was seen in hamsters.

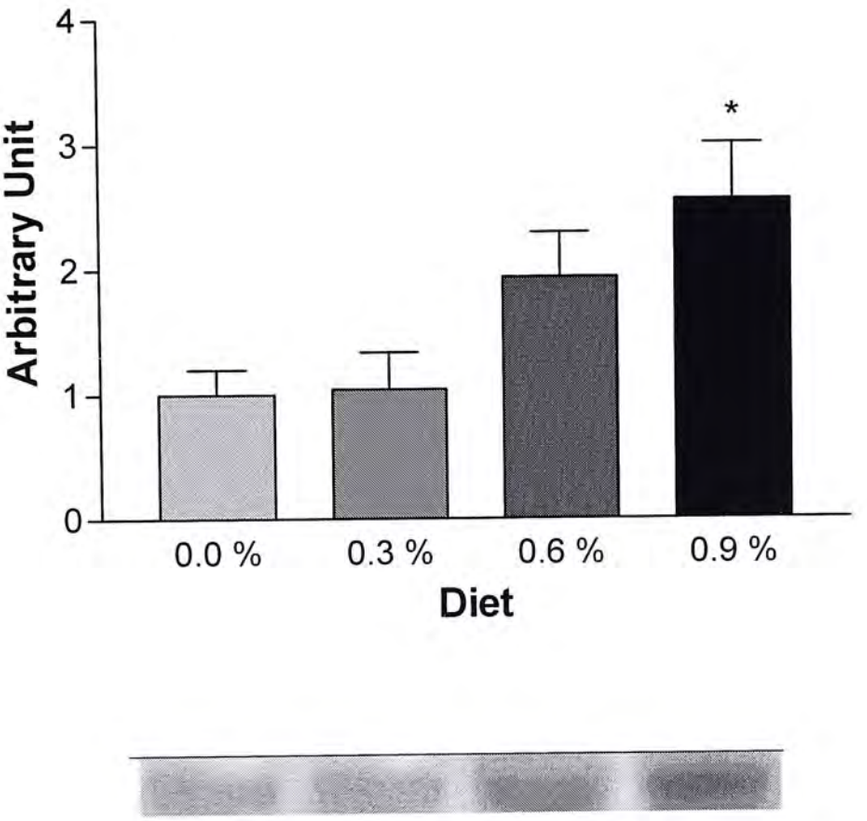

Figure 3.6. Representative Western blot and group data depicting CYP7A1 abundance in the liver of Sprague-Dawley rats fed the control diet (0.0%), 0.3%, 0.6 and 0.9% cholesterol diet for one month. Data are mean±S.E.M., n=5. * $P<0.05$ when compared with the control.

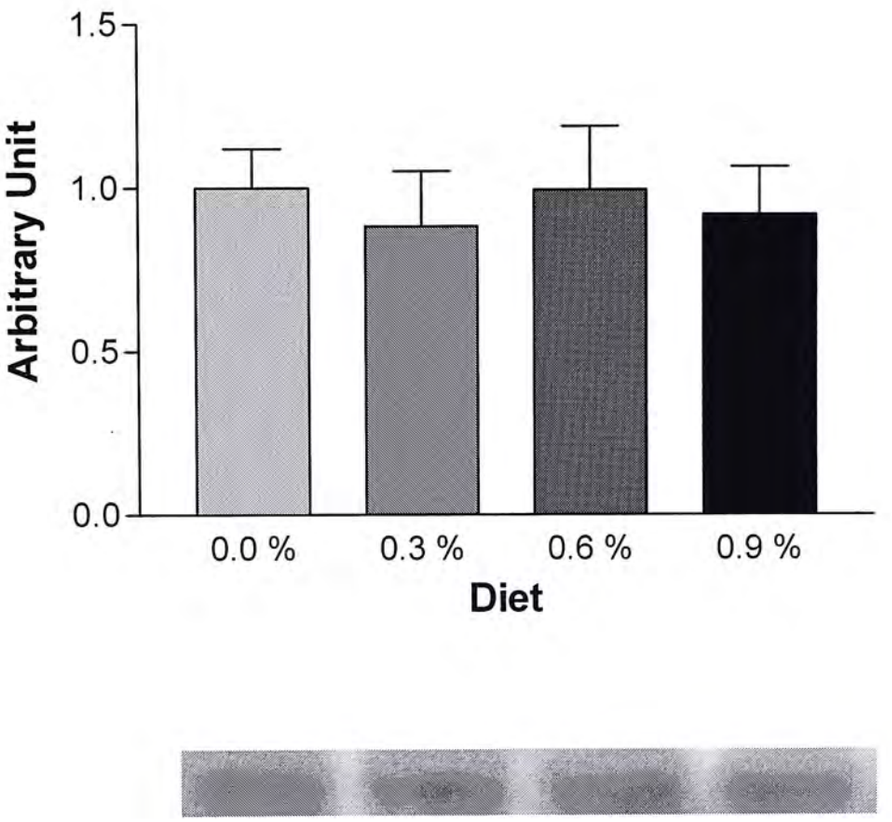

Figure 3.7. Representative Western blot and group data depicting CYP7A1 abundance in the liver of Golden Syrian hamsters fed the control diet (0.0%), 0.3%, 0.6 and 0.9% cholesterol diet for one month. Data are mean±S.E.M., n=5.

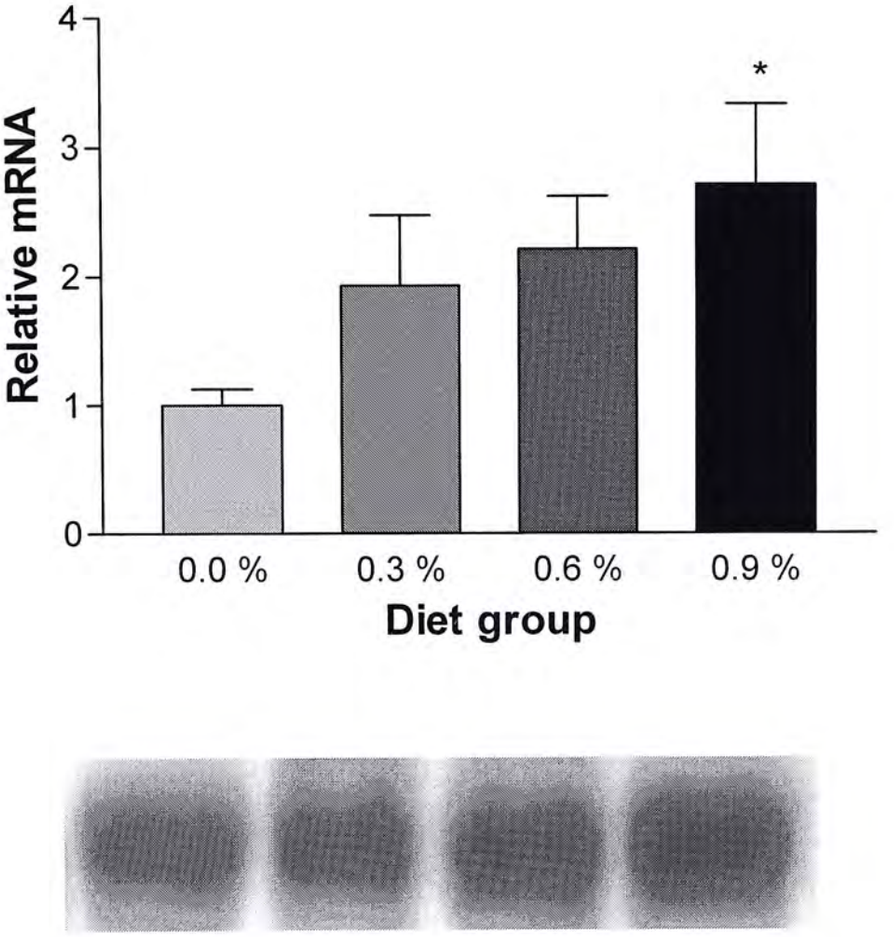

Figure 3.8. Representative Northern blot and group data depicting CYP7A1 mRNA abundance in the liver of Sprague-Dawley rats fed the control diet (0.0%), 0.3%, 0.6 and 0.9% cholesterol diet for one month. Data are mean±S.E.M., n=5. * $P<0.05$ when compared with the control.

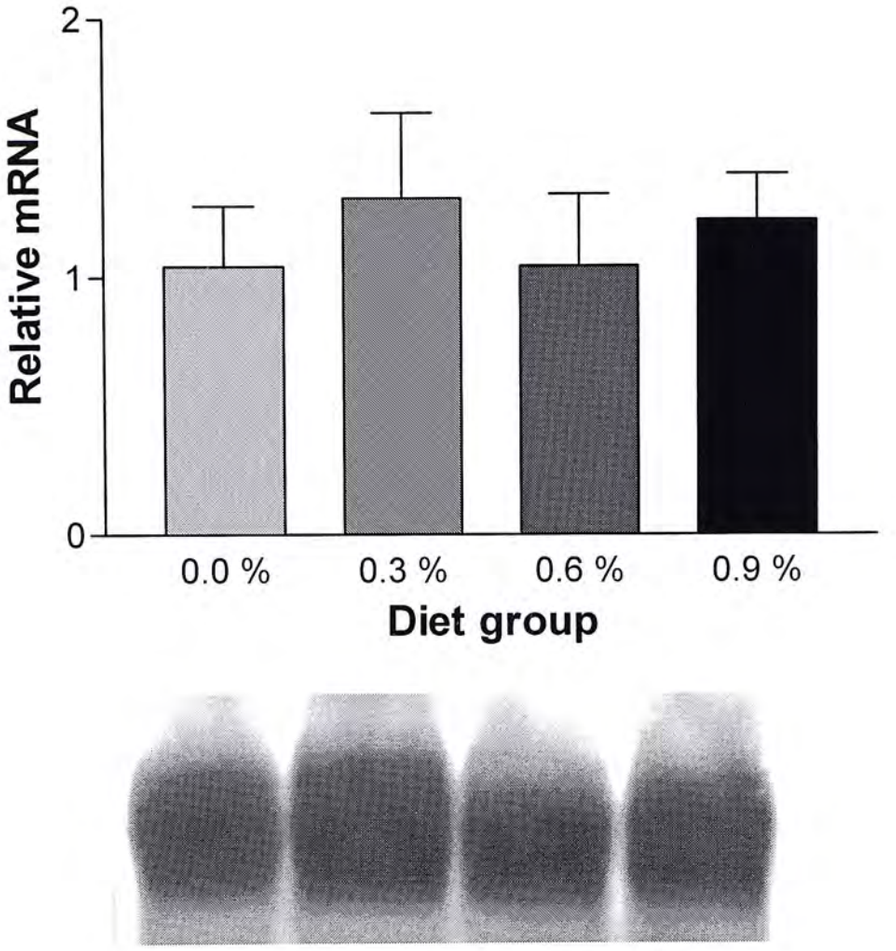

Figure 3.9. Representative Northern blot and group data depicting CYP7A1 mRNA abundance in the liver of Golden Syrian hamsters fed the control diet (0.0%), 0.3%, 0.6 and 0.9% cholesterol diet for one month. Data are mean±S.E.M., n=5.

3.4.5 LXR-α demonstrated a parallel changes in its expression at both translational and transcriptional level

LXR-α is the transcription factor of CYP7A1. Figure 3.10 showed the immunoblots and group results of LXR-α protein in rats fed various cholesterol diets. It was found that LXR-α protein expression increased with the increasing dietary cholesterol in rats. However, the LXR-α protein was not detectable in hamsters.

The mRNA expression level of LXR-α in both rats and hamsters was determined, using GAPDH to normalize the uneven loading of samples. Figure 3.11 showed the photo and group results of the LXR-α mRNA expression in liver of rats fed various cholesterol diets for a month, while Figure 3.12 for that of hamsters. In rats, the mRNA expression of LXR-α was up-regulated in accordance to the increasing cholesterol in the diet though not statistically significant. In contrast, no changes were found among all the diet groups in hamsters.

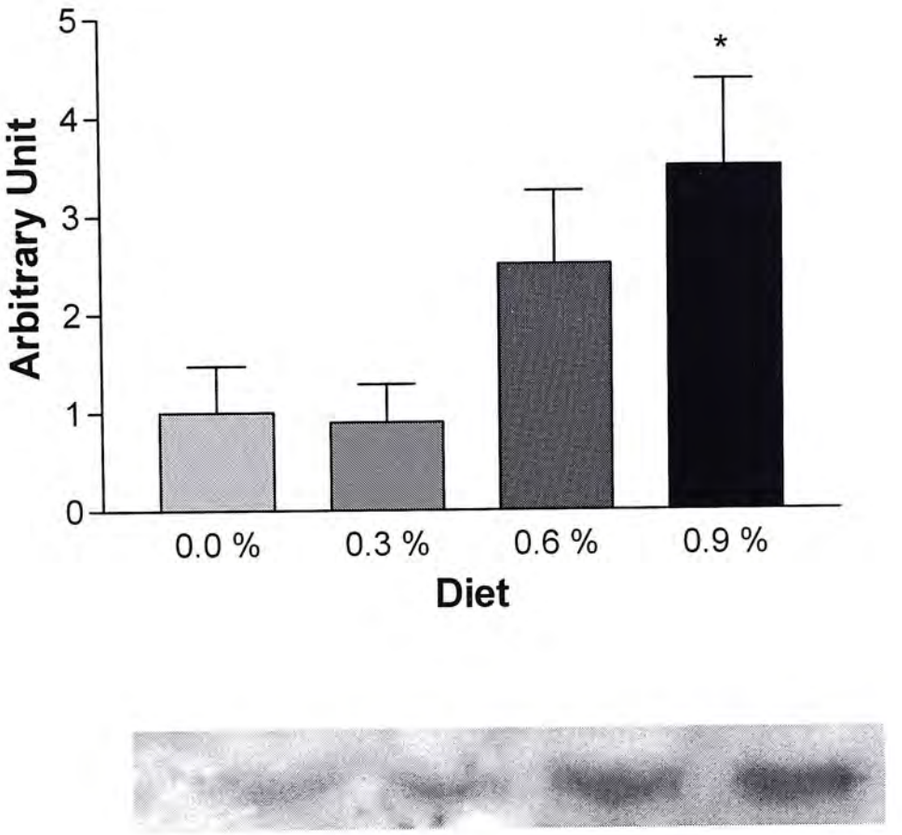

Figure 3.10. Representative Western blot and group data depicting LXR-α abundance in the liver of Sprague-Dawley rats fed the control diet (0.0%), 0.3%, 0.6 and 0.9% cholesterol diet for one month. Data are mean±S.E.M., n=5. * $P<0.05$ when compared with the control.

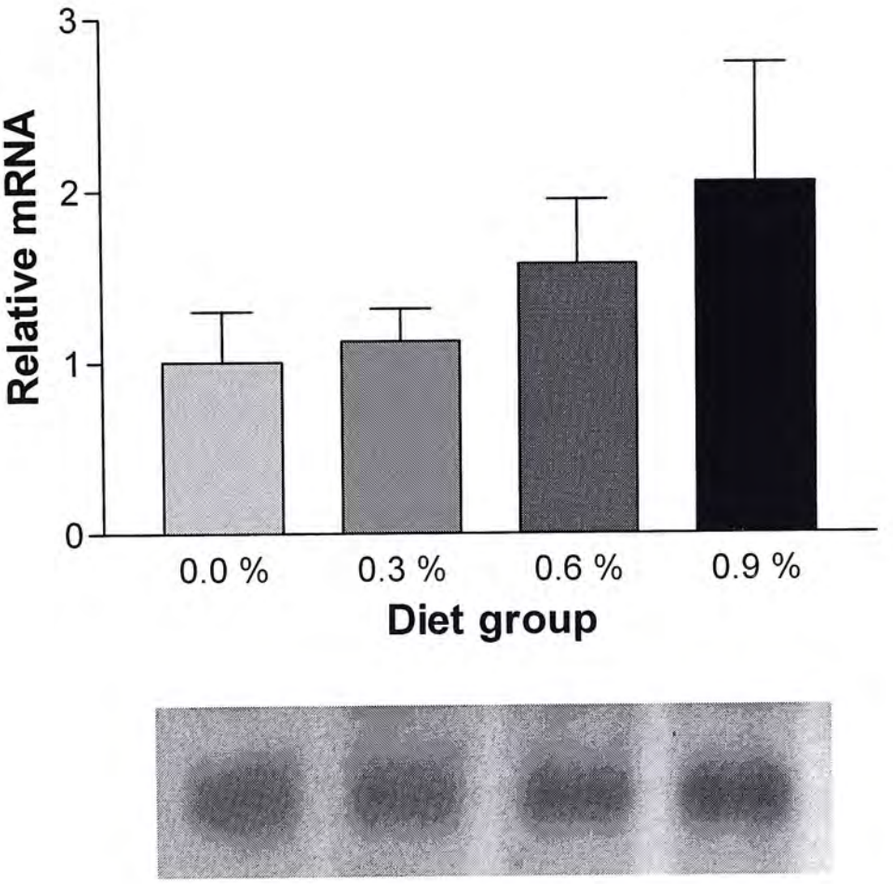

Figure 3.11. Representative Northern blot and group data depicting LXR-α mRNA abundance in the liver of Sprague-Dawley rats fed the control diet (0.0%), 0.3%, 0.6 and 0.9% cholesterol diet for one month. Data are mean±S.E.M., n=5.

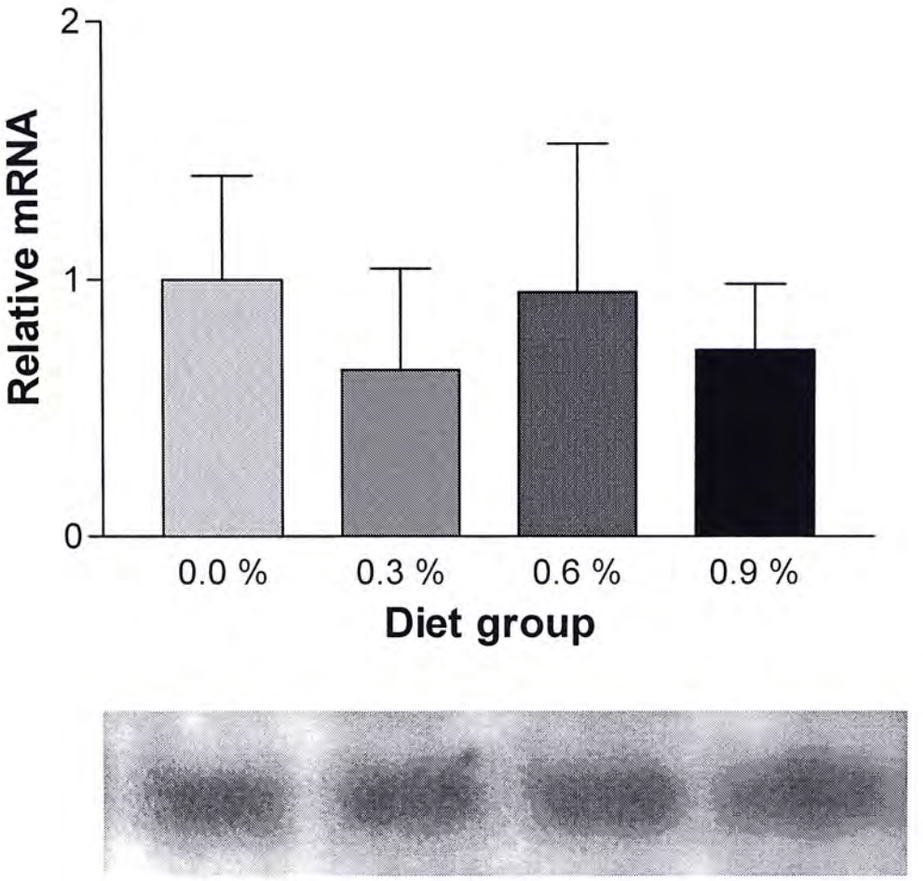

Figure 3.12. Representative Northern blot and group data depicting LXR-α mRNA abundance in the liver of Golden Syrian hamsters fed the control diet (0.0%), 0.3%, 0.6 and 0.9% cholesterol diet for one month. Data are mean±S.E.M., n=5.

3.5 Discussion

The present results clearly demonstrated that rats eliminated hepatic cholesterol more efficiently than hamsters. This was supported by two evidences. First, with increasing dietary cholesterol, rats excreted more fecal total neutral sterols and bile acids while hamsters only had minor increase in fecal total neutral sterols. Second, rats excreted more fecal total neutral sterols and bile acids than hamsters in the same diet group. The level of fecal total bile acids is closely related to the responsiveness to dietary cholesterol. A study conducted by Beynen et al. (1989) showed rabbits that were hypo-responsive to dietary cholesterol excreted more fecal bile acids than those hyper-responders. Therefore efficient elimination of cholesterol and bile acids was, at least partly, responsible for the hypo-responsive to dietary cholesterol in rats while the hyper-responsiveness in hamsters was due to inefficient removal of excess cholesterol in feces.

There are two mechanisms responsible for the increased fecal bile acids excretion with increasing dietary cholesterol in rats: increased bile acids biosynthesis and inefficient bile acids reabsorption from the intestine via the enterohepatic circulation. The current study supported the view that increased bile acids biosynthesis was accounted for the increased fecal bile acids. This is because CYP7A1, the rate-determining enzyme which controls the conversion of hepatic cholesterol to bile acids (Russell 2003; Russell et al., 1992), was up-regulated in response to higher dietary cholesterol in rats. However, the increased biosynthesis of bile acids could not be solely responsible for the increased fecal bile acids because enterohepatic circulation reabsorbed bile acids from intestine to the liver. If the enterohepatic circulation was efficient in rats, most of bile acids would be returned back to the liver but did not stay in the feces, as observed. It was suggested that

malabsorption of bile acids in rats responsible for the increased fecal bile acids (Horton et al., 1995; Bjorkhem et al., 1991). The observation of increased both fecal neutral sterols and bile acids in rats with increasing dietary cholesterol content suggested that the increased fecal neutral sterols might bind the bile acids in the intestine, hindering the reabsorption of bile acids to the liver. In fact, efficient removal of bile acids in feces further assists the up-regulation of CYP7A1 because bile acids inhibit the expression of CYP7A1 (Bjorkhem et al., 1991; Parks et al., 1991). Hence, it was suggested that both malaborption of bile acids and increased expression of CYP7A1, which were inter-related, were responsible for the increased fecal bile acids in rats.

In contrast, unchanged fecal bile acids level was observed in hamsters in response to increasing dietary cholesterol. The finding that CYP7A1 protein expression was not increased in response to the higher dietary cholesterol content in hamsters was responsible for the differences between rats and hamsters. In fact, different effect of dietary cholesterol on CYP7A1 expression/activity was observed by Ando et al. (2005). Rats and mice showed a marked up-regulation whereas hamsters, monkeys, humans, guinea pigs, and rabbits did not respond or even repress CYP7A1 expression with the increasing dietary cholesterol (Horton et al., 1995; Dueland et al., 1993; Nguyen et al., 1999). The mechanism for the unchanged CYP7A1 protein expression in hamsters was supposed to be the opposite of rats, which had increased CYP7A1 protein expression in response to dietary cholesterol. As enterohepatic circulation was efficient in hamsters, more bile acids were reabsorbed back to the liver, which inhibit the expression of CYP7A1. Hence, both unchanged fecal bile acids and hepatic CYP7A1 protein expression were observed in hamsters with increasing dietary cholesterol content.

This study further investigated whether any other factors involved in the

up-regulation of CYP7A1 in rats and unchanged expression of CYP7A1 in hamsters. It has been demonstrated in previous studies that LXR-α can up-regulate the expression of CYP7A1 (Chiang et al., 2001; Gupta et al., 2002), hence, we looked into the effect of dietary cholesterol on LXR-α. The results showed that with increasing dietary cholesterol, up-regulation of LXR-α was observed in rats but not in hamsters. In addition, a parallel up-regulation of LXR-α and CYP7A1 at both mRNA/protein expression was observed in rats. Therefore, increase in CYP7A1 protein and mRNA expression in response to the increasing dietary cholesterol in rats was controlled by LXR-α, at transcriptional level. The regulatory mechanism of LXR-α was demonstrated in one *in vitro* study showing that ligand bound LXR-α up-regulated CYP7A1 by binding to the LXRE in the CYP7A1 promoter region (Lehmann et al., 1997). Further evidence was obtained in the experiment with the analysis of LXR-α knock-out mice (LXR-$\alpha^{-/-}$), which could not up-regulate CYP7A1 in response to cholesterol feeding (Peet et al., 1998b). On the other hand, LXR-α was not up-regulated in hamsters with increasing dietary cholesterol. Therefore, in addition to the malabsorption of bile acids, which inhibit the expression of CYP7A1, the up-regulation of CYP7A1 in rats was related to increased expression of LXR-α. On the other hand, the unchanged CYP7A1 expression in hamsters was due to the unchanged level of LXR-α in response to increasing dietary cholesterol and feedback regulation of bile acids.

To summarize, rats were hypo-responsive to dietary cholesterol because they eliminated cholesterol efficiently. This was controlled by malabsorption and increased biosynthesis of bile acids. The biosynthesis of bile acids was increased because the expression of CYP7A1 was increased. The CYP7A1 expression was controlled at transcriptional level by its positive regulator, LXR-α, which increased in response to increasing dietary cholesterol. As a result, more hepatic cholesterol was converted to

bile acids in rats in response to dietary cholesterol (Figure 3.13). This pathway eliminated excess hepatic cholesterol and provided more space for further uptake of cholesterol from blood, via the LDL-receptor pathway, as demonstrated in Chapter 2. In contrast, hamsters were hyper-responsive to dietary cholesterol because their CYP7A1 and LXR-α expression did not respond significantly to any changes in dietary cholesterol content in the diet. As a result, excess cholesterol could not be removed from the liver and this hindered the uptake of cholesterol from blood, leading to a sharp increase in serum cholesterol level when dietary cholesterol was increased to 0.9% (Figure 3.14).

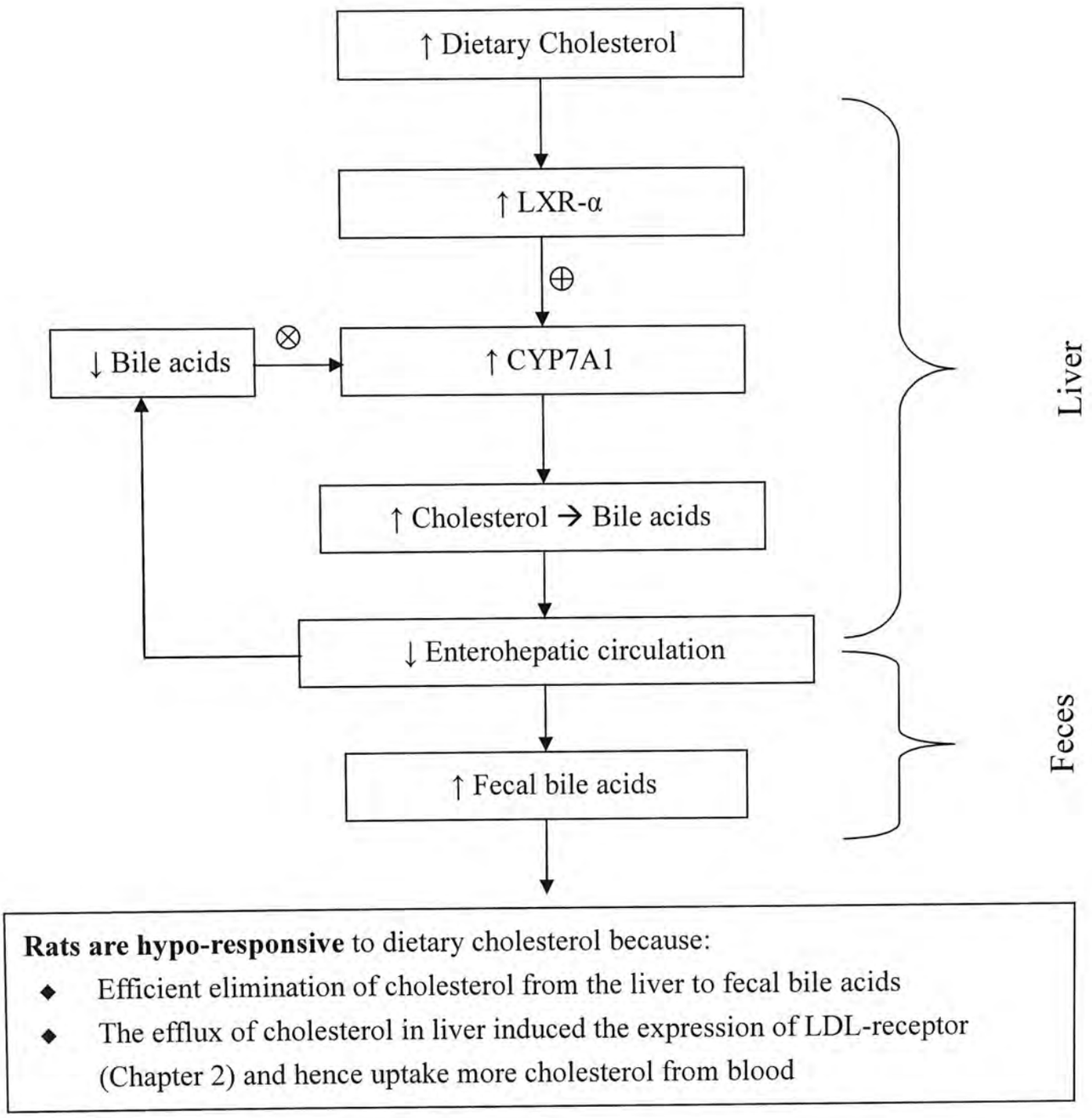

Figure 3.13. Illustration of the hypo-responsive mechanism of rats in response to a high dietary cholesterol.

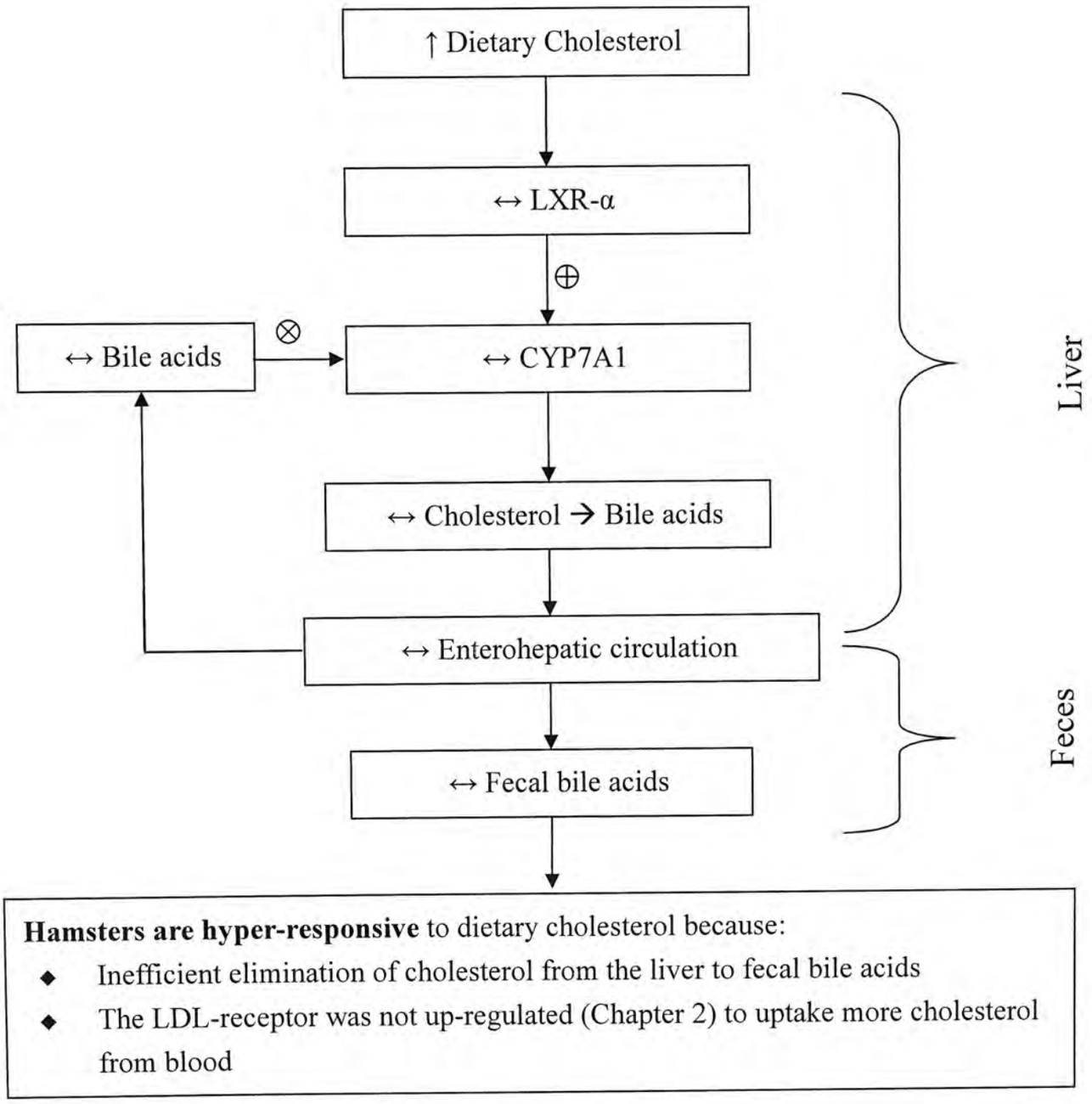

Figure 3.14. Illustration of the hyper-responsive mechanism of hamsters in response to a high dietary cholesterol.

CHAPTER 4

Mechanism for individual variation of serum cholesterol level in rats and hamsters fed a high cholesterol diet

4.1 Introduction

The previous two chapters investigated the effect of a high cholesterol diet on blood cholesterol level in rats and hamsters. Briefly, rats were hypo-responsive to a high cholesterol diet because the protein expression of LDL-receptor and CYP7A1 was up-regulated in response to a high cholesterol level in the diet. As a result of the efficient elimination of cholesterol from the liver and increased uptake of cholesterol from blood, rats could therefore tolerate high dietary cholesterol. In contrast, hamsters were hyper-responsive to a high cholesterol diet. This was because the protein expression of LDL-receptor and CYP7A1 did not respond to the variation of cholesterol content in the diet. Hence, hamsters accumulated excess dietary cholesterol and led to a sharp increase in serum cholesterol in response to a high cholesterol diet.

It is well-known that dietary cholesterol raises the average serum cholesterol concentration of a population (Ginsberg et al., 1995; Jones et al., 1994). For example, the study by Jones et al. (1994) has demonstrated that blood cholesterol is raised by 4.1mg/dl per every 100mg dietary cholesterol. However, not all individuals within the population are responsive in a same way to a high dietary cholesterol (Hegsted et al., 1986; Glatz et al., 1993; Adrens et al., 1957; Herron et al., 2003). Instead, a great variability in response was found. Some individuals are hyper-responders to a high

dietary cholesterol and develop a sharp increase in blood cholesterol, while some are hypo-responders (Howell et al., 1997; Herron et al., 2003). McNamara et al. (2000) have estimated that about 15-25% of the population is sensitive to a high cholesterol diet. This has aroused our interest in investigating the underlying mechanism.

4.2 Objective

This study was performed to determine individual variation in expression of CYP7A1, LDL-receptor, nSREBP-2, LXR-α and HMG-CoA reductase in response to a same amount of dietary cholesterol, using rats and hamsters as models. The information obtained would help explain why some are hyper- while some are hypo-responsive to dietary cholesterol within the same species.

4.3 Methods and materials

4.3.1 Diet and animals

Sprague-Dawley rats and Golden Syrian hamsters were housed in colony cages in a room with controlled temperature (20-23℃) and lighting (alternating 12 hour periods of light and dark). Prior to the studies, the animals were fed a pelletized commercial nonpurified diet for 6 days after arrival. Afterwards, the animals were switched to a 0.9% high cholesterol diet as previously described in Chapter 2 and 3.

Thirty rats (190 – 240g) and thirty hamsters were fed a high cholesterol semisynthetic diet *ad libitum* for 4 weeks. The animals were killed by a nitrogen gas tank during the mid-dark phase of the light cycle. The liver was rapidly removed and frozen in liquid nitrogen. Serum total cholesterol (TC) levels were determined enzymatically by the use of commercial kits (Sigma Chemical, St Louis, MO, USA).

4.3.2 Western blot

The protein extraction and Western blot were carried out with the procedures previously described in Chapter 2 and 3.

4.3.3 Statistics

For the serum cholesterol, the statistical significance of differences between groups was assessed by Student's *t*-test, using Prism® (Graphpad software). Differences between groups were considered significant when $P<0.05$. In addition, the correlation between protein expression and serum cholesterol in the animal models were assessed by correlation analysis, using Prism® (Graphpad software). P value and coefficient of determinant (r^2) were measured and the result considered as statistically significant

when $P < 0.05$.

4.4 Results

4.4.1 Growth and food intake

Table 4.1 showed the changes in body weight and food intake of rats and hamsters after one month feeding of a high cholesterol diet. The rats had gained 91 grams while the hamsters had body weight gained only 4 grams. This was in agreement with the observation that the rats consumed 31 grams diet while hamsters only had a food intake of 7 grams per day.

4.4.2 Change of serum cholesterol

Figure 4.1 showed the change in serum total cholesterol (TC) of rats after feeding rats with a 0.9% cholesterol diet for one month. The serum TC of rats increased from 76.2±2.4 to 93.8±4.9mg/dl ($P<0.01$), which represented only 23% increase. In contrast, the serum TC of hamsters (Figure 5.2) increased from 110.8±2.1 to 332.9±9.7mg/dl ($P<0.0001$), which represented 200% increase (Figure 4.2).

4.4.3 Correlation between various protein expression and serum cholesterol

4.4.3.1 Correlation between LDL-receptor and serum total cholesterol in rats

Figure 4.3 showed the relative LDL-receptor protein expression against serum total cholesterol in rats. The coefficient of determinant (r^2) was 0.0044 and the P value was larger than 0.72, suggesting that there had no correlation between the LDL-receptor expression and serum TC in rats fed a high cholesterol diet.

4.4.3.2 Correlation between CYP7A1 and serum total cholesterol in rats

Figure 4.4 showed the correlation between relative CYP7A1 protein expression and serum TC in rats. The coefficient of determinant (r^2) was 0.3351 and $P<0.001$, suggesting that the CYP7A1 expression was negatively correlated with serum TC

Table 4.1. The changes in body weight and food intake in rats and hamsters fed a 0.9% cholesterol diet for 1 month. n = 30 in each group. Data are mean ± S.E.M.

	Rat	Hamster
Initial body weight (grams/ day/animal)	249 ± 6	108 ± 2
Final body weight (grams/ day/animal)	340 ± 6	112 ± 1
Daily food intake (grams/ day/animal)	31 ± 4	7 ± 1

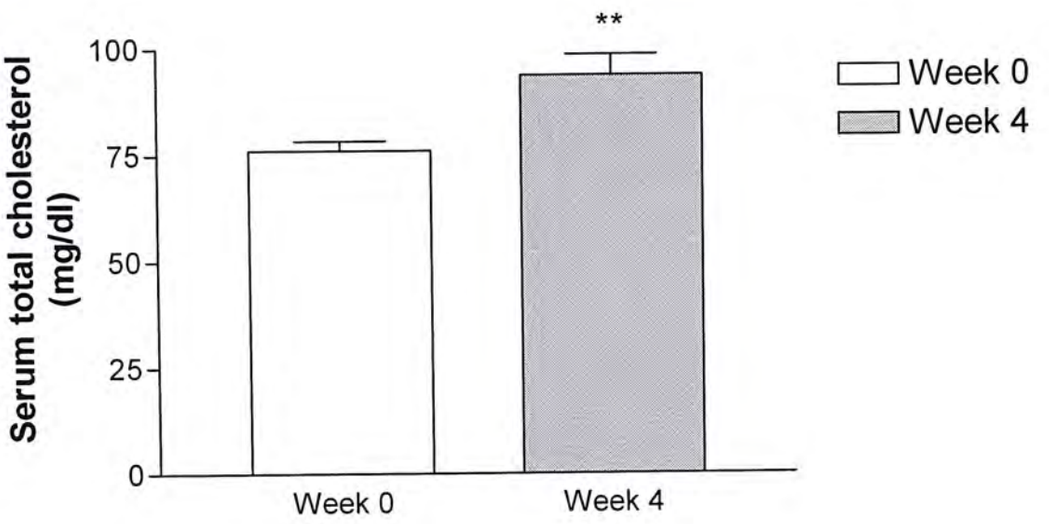

Figure 4.1. The serum total cholesterol of Sprague-Dawley rats fed a 0.9% cholesterol diet for one month. Data are mean±S.E.M.; n=30; ** $P<0.01$ when compared with week 0.

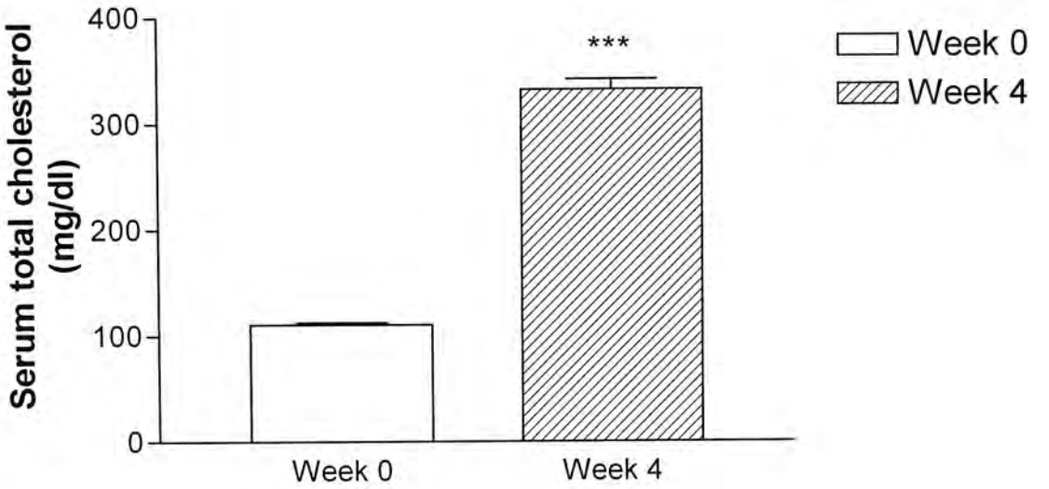

Figure 4.2. The serum total cholesterol of Golden Syrian hamsters fed a 0.9% cholesterol diet for one month. Data are mean±S.E.M.; n=30; *** $P<0.0001$ when compared with week 0.

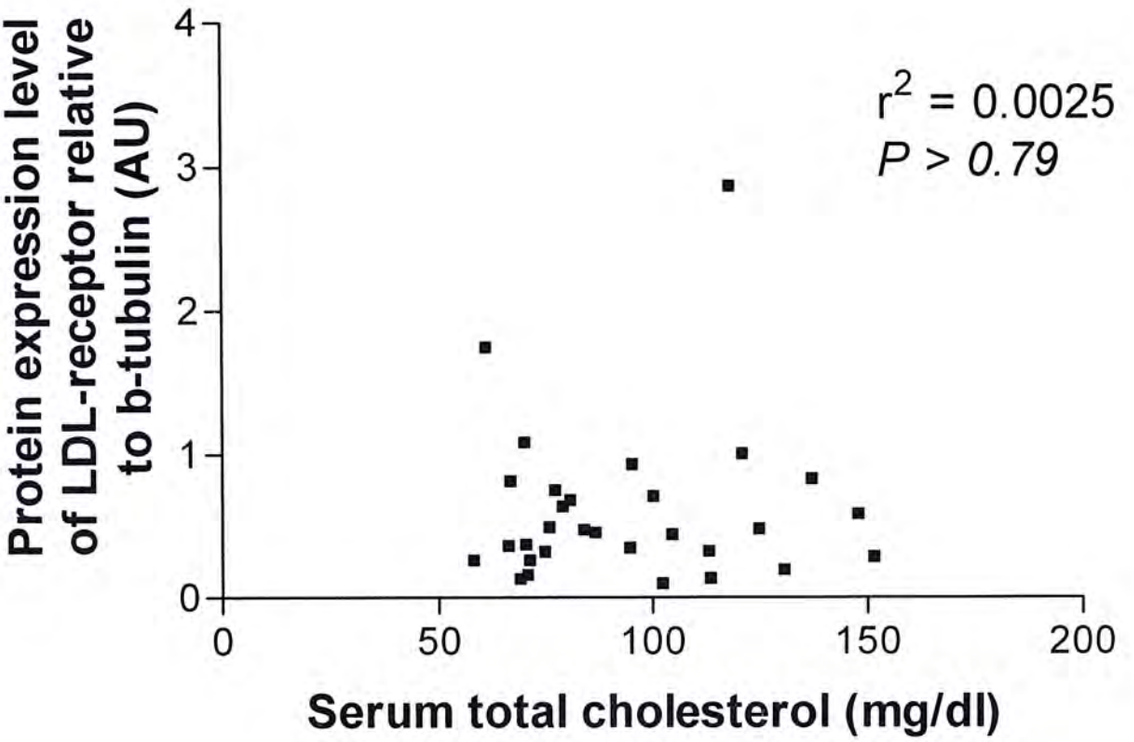

Figure 4.3. Correlation between the relative protein expression of LDL-receptor and serum total cholesterol in rats fed a 0.9 % high cholesterol diet for a month. The coefficient of determinant (r^2) and the P value were shown on the graph.

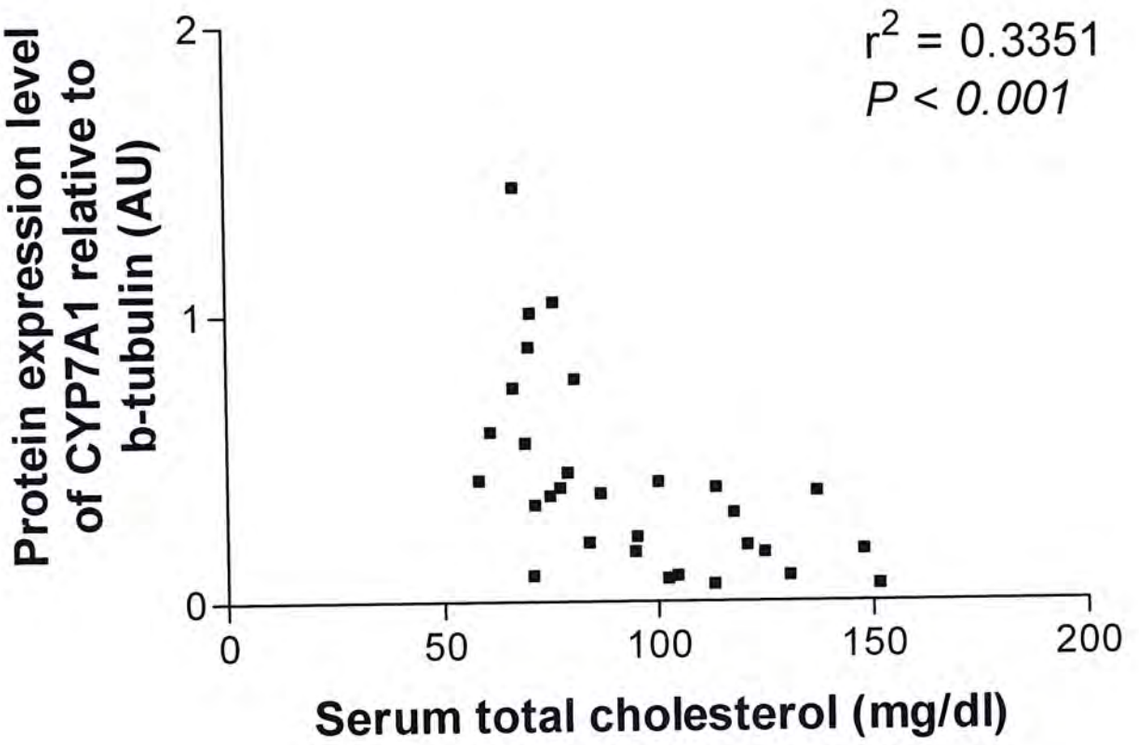

Figure 4.4. Correlation between the relative protein expression of CYP7A1 and serum total cholesterol in rats fed a 0.9 % high cholesterol diet for a month. The coefficient of determinant (r^2) and the P value were shown on the graph.

level in rats fed a high cholesterol diet.

4.4.3.3 Correlation between nSREBP-2 and serum total cholesterol in rats

Figure 4.5 showed the relative nSREBP-2 protein expression against serum total cholesterol in rats. The coefficient of determinant (r^2) was 0.0224 and the P value was larger than 0.43, suggesting that there had no correlation between the nSREBP-2 expression and serum TC in rats fed a high cholesterol diet.

4.4.3.4 Correlation between LXR-α and serum total cholesterol in rats

Figure 4.6 showed the relative LXR-α protein expression against serum total cholesterol in rats. The coefficient of determinant (r^2) was 0.0002 and the P value was larger than 0.94, suggesting that there had no correlation between the nSREBP-2 expression and serum TC in rats fed a high cholesterol diet.

4.4.3.5 Correlation between HMG-CoA reductase and serum total cholesterol in rats

Figure 4.7 showed the relative HMG-CoA reductase protein expression against serum total cholesterol in rats. The coefficient of determinant (r^2) was 0.0092 and the P value was larger than 0.61, suggesting that there had no correlation between the nSREBP-2 expression and serum TC in rats fed a high cholesterol diet.

4.4.3.6 Correlation between LDL-receptor and serum total cholesterol in hamsters

Figure 4.8 showed the correlation between the relative LDL-receptor protein expression and serum TC in hamsters. The coefficient of determinant (r^2) was 0.3785 and the $P<0.05$, indicating that the LDL-receptor expression was negatively correlated with serum TC in hamsters.

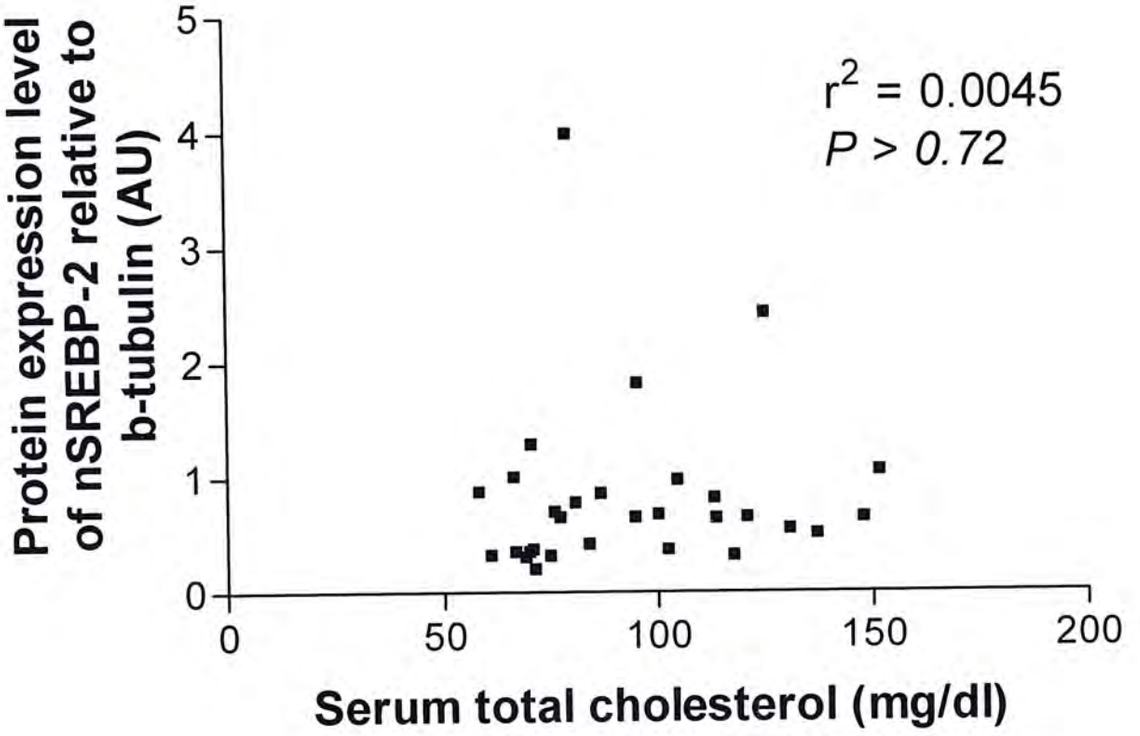

Figure 4.5. Correlation between the relative protein expression of nSREBP-2 and serum total cholesterol in rats fed a 0.9 % high cholesterol diet for a month. The coefficient of determinant (r^2) and the P value were shown on the graph.

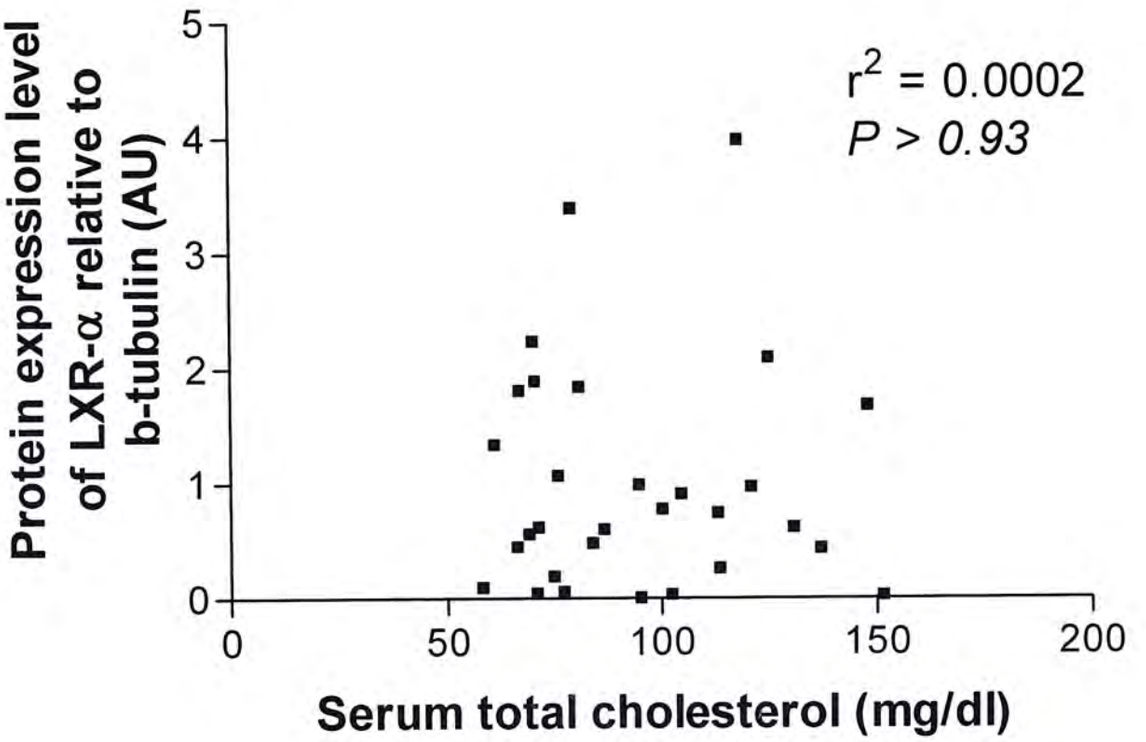

Figure 4.6. Correlation between the relative protein expression of LXR-α and serum total cholesterol in rats fed a 0.9 % high cholesterol diet for a month. The coefficient of determinant (r^2) and the P value were shown on the graph.

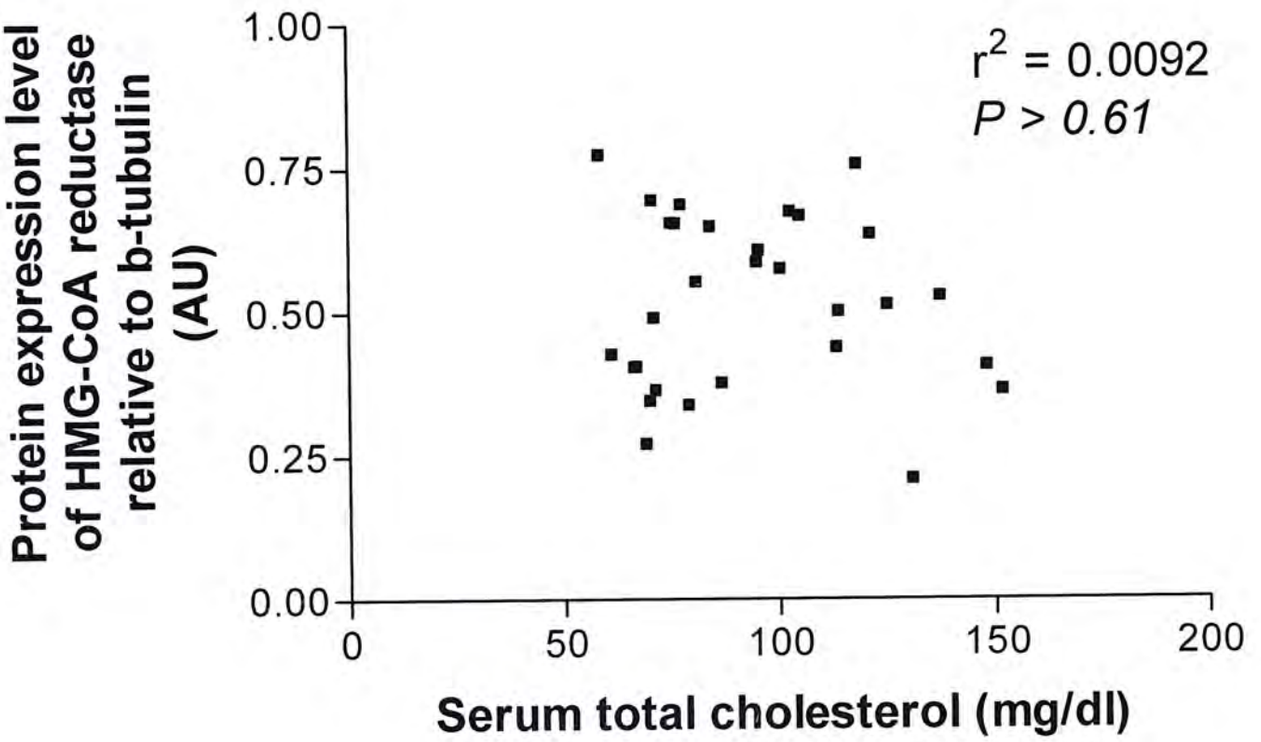

Figure 4.7. Correlation between the relative protein expression of HMG-CoA reductase and serum total cholesterol in rats fed a 0.9 % high cholesterol diet for a month. The coefficient of determinant (r^2) and the P value were shown on the graph.

4.4.3.7 Correlation between CYP7A1 and serum total cholesterol in hamsters

Figure 4.9 showed the relative CYP7A1 protein expression in relation to serum TC in hamsters. The coefficient of determinant (r^2) was 0.5556 and $P<0.05$. The present result demonstrated that the CYP7A1 expression was inversely correlated with serum TC in hamsters.

4.4.3.8 Correlation between nSREBP-2 and serum total cholesterol in hamsters

Figure 4.10 showed the relative nSREBP-2 protein expression in relation to serum TC in hamsters. The coefficient of determinant (r^2) was 0.0906 and the P value was larger than 0.11, suggesting that there had no correlation between the nSREBP-2 expression and serum TC in hamsters fed a high cholesterol diet

4.4.3.9 Correlation between HMG-CoA reductase and serum total cholesterol in hamsters

Figure 4.11 showed the relative HMG-CoA reductase protein expression in relation to serum TC in hamsters. The coefficient of determinant (r^2) was 0.0631 and the P value was larger than 0.19. The present result demonstrated that the HMG-CoA reductase expression was inversely correlated with serum TC in hamsters.

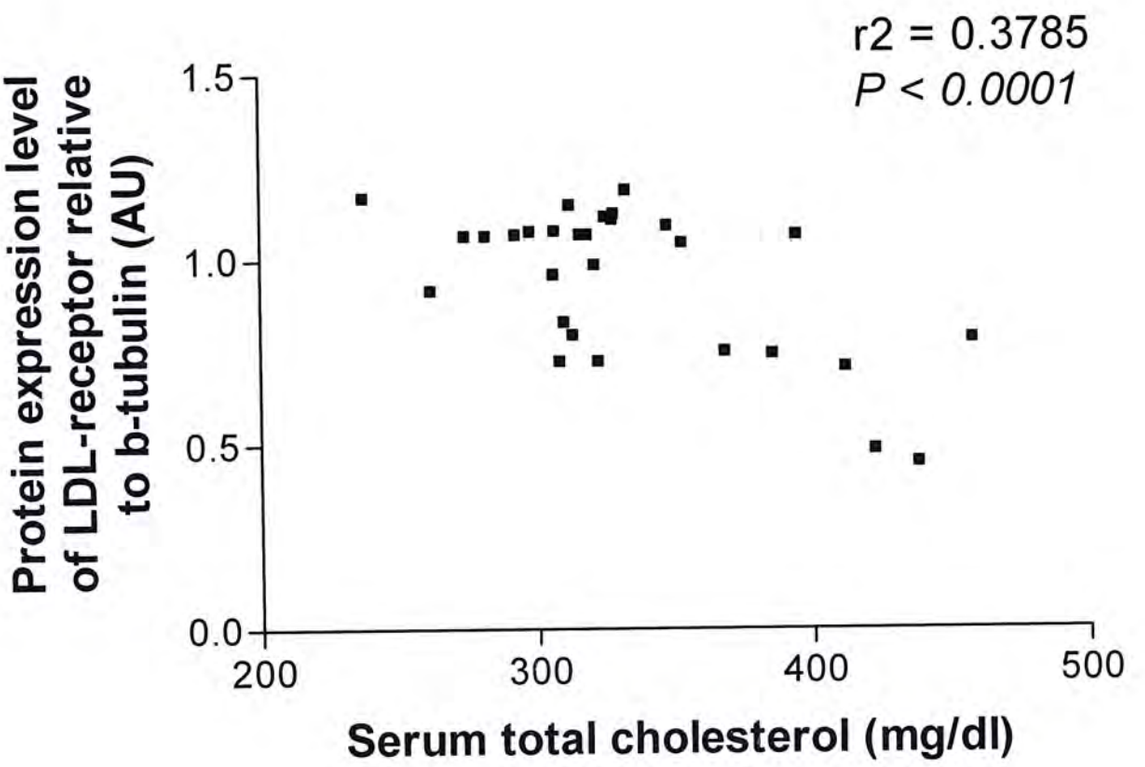

Figure 4.8. Correlation between the relative protein expression of LDL-receptor and serum total cholesterol in hamsters fed a 0.9 % high cholesterol diet for a month. The coefficient of determinant (r^2) and the P value were shown on the graph.

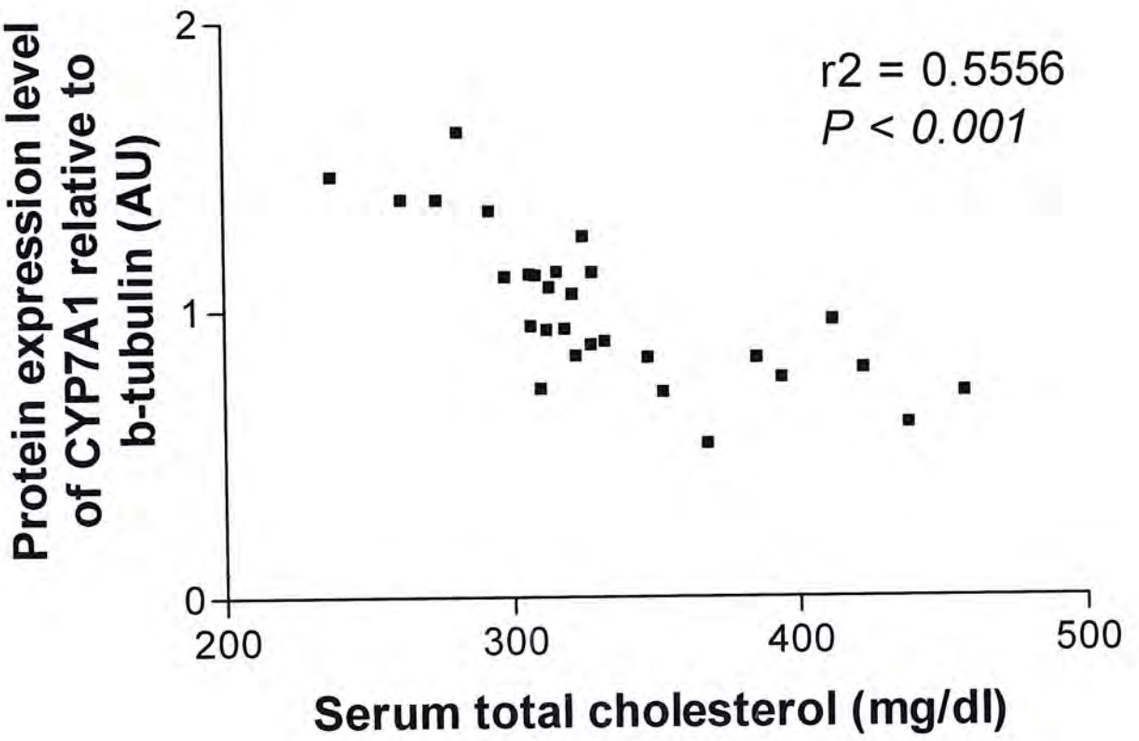

Figure 4.9. Correlation between the relative protein expression of CYP7A1 and serum total cholesterol in hamsters fed a 0.9 % high cholesterol diet for a month. The coefficient of determinant (r^2) and the P value were shown on the graph.

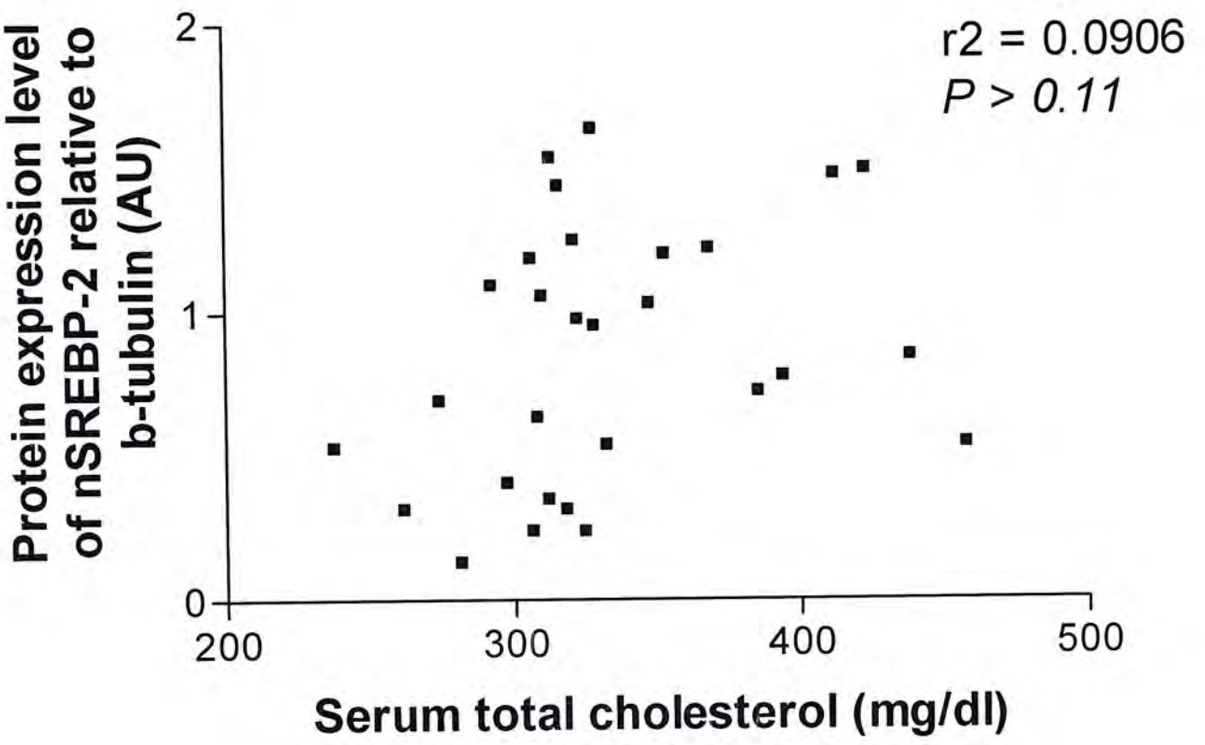

Figure 4.10. Correlation between the relative protein expression of nSREBP-2 and serum total cholesterol in hamsters fed a 0.9 % high cholesterol diet for a month. The coefficient of determinant (r^2) and the P value were shown on the graph.

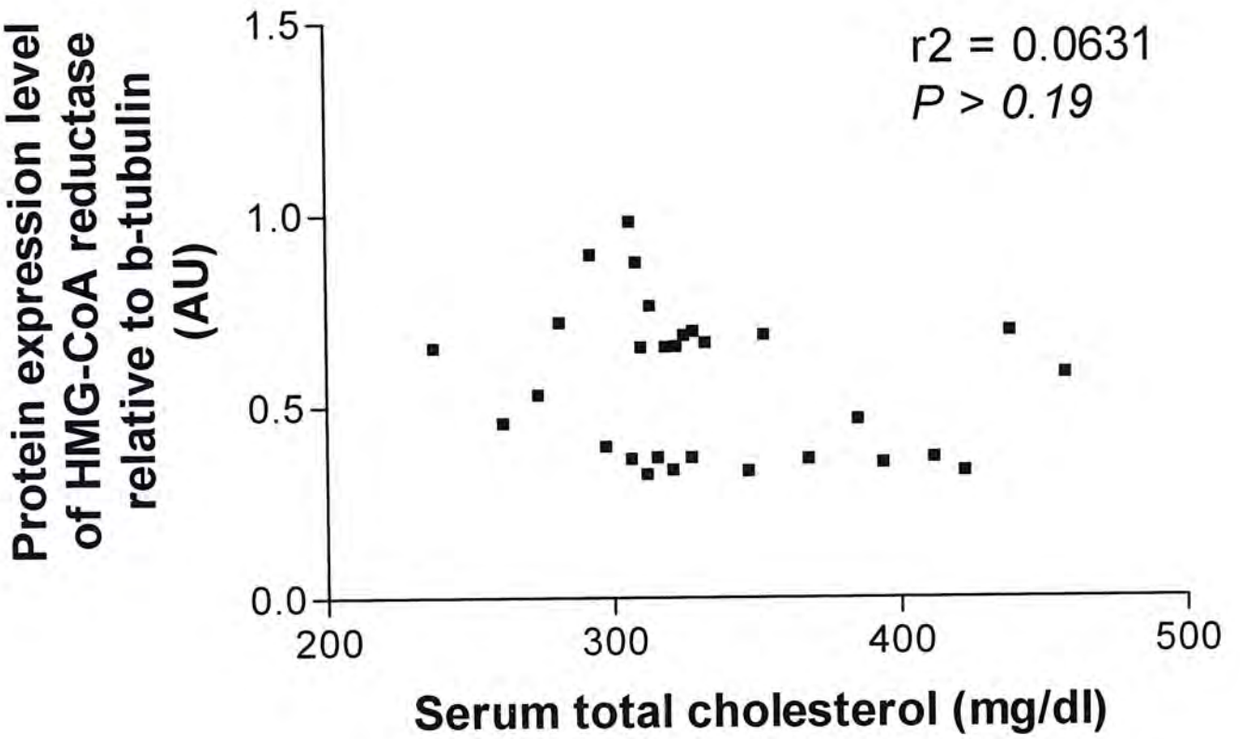

Figure 4.11. Correlation between the relative protein expression of HMG-CoA reductase and serum total cholesterol in hamsters fed a 0.9 % high cholesterol diet for a month. The coefficient of determinant (r^2) and the P value were shown on the graph.

4.5 Discussion

The present study clearly demonstrated that elevation of dietary cholesterol raised blood cholesterol in both hamsters and rats. However, serum TC increased only by 22% in rats while it raised by 200% in hamsters. In general, elevation of serum TC in hamsters was 12-fold higher than that in rats fed the same high cholesterol diet. The previous two chapters had examined the molecular mechanism by which why two species had their blood cholesterol responded differently to the same high dietary cholesterol. The present study was, however, to carry out further on a fundamental issue of why some individuals were hyper-responsive while others were hypo-responsive to dietary cholesterol within a species.

Individual rats responded differently to the dietary cholesterol. As shown in Figures 4.3, serum TC ranged from 58 to 152mg/dl was observed. When the relationship between the relative expression of LDL-receptor and serum cholesterol was examined (Figure 4.3), no correlation was found, suggesting that the LDL-receptor protein expression is not a limiting factor in regulation of serum cholesterol level in rats fed the same level of dietary cholesterol. The present study found an inverse correlation between relative CYP7A1 protein expression and the level of serum cholesterol in rats (Figure 4.4). For those individuals with lower CYP7A1 expression, the serum total cholesterol was higher. The reverse was true for those individuals with higher protein expression of CYP7A1 had lower serum total cholesterol. The present results indicated that the individual variation in blood cholesterol level of rats fed the same amount of dietary cholesterol is dependent at least partially on their variation in CYP7A1 expression. Further investigation on the correlation between the expressions of nSREBP-2, LXR-α, HMG-CoA reductase and serum cholesterol were examined (Figure 4.5 to 4.7). No correlation was found

between the expression of these proteins and the level of serum cholesterol, suggesting that the nSREBP-2, LXR-α and HMG-CoA reductase protein expressions were not the limiting factors in controlling the serum cholesterol in rats fed a high dietary cholesterol.

Hamsters demonstrated a sharp increase in serum TC on a high cholesterol diet for a month. However, individual variation in blood cholesterol was so significant with a range of 236 to 457mg/dl (Figure 4.8). Interestingly, an inverse correlation was observed between LDL-receptor protein expression and serum cholesterol level in hamsters (Figure 4.8). Similarly, CYP7A1 protein expression was also inversely correlated with serum cholesterol level (Figure 4.9). The efficient elimination of cholesterol from blood is mediated by LDL-receptor and subsequently via CYP7A1 (classical) pathway, which converts hepatic cholesterol to bile acids. The present study demonstrated that both the LDL-receptor and CYP7A1 were the regulatory points that were responsible for controlling the blood cholesterol in hamsters. However, no correlation between the protein expression of nSREBP-2, HMG-CoA reductase and serum total cholesterol was found (Figure 4.10 and 4.11). It was concluded that the hyper-responsive hamsters was due to lower expression of both LDL-receptor and CYP7A1 while the hypo-responsive ones were due to higher expression of these two proteins in hamsters on the same high cholesterol diet.

The variation in expression of LDL-receptor and CYP7A1 may be attributable to the DNA polymorphism. One relevant clinical study conducted in Czech indicated that polymorphism in CYP7A1 promoter region was closed linked to some hyper-responsive male individuals (Hubacek et al., 2003). For those individuals with CYP7A1 homozygous -204A allele, which is a polymorphism in the promoter region of CYP7A1, their blood cholesterol level demonstrated a minimal response to dietary cholesterol. In contrast, lowering effect on total blood cholesterol was observed in CC

homozygotes in response to dietary cholesterol. In addition, several studies also indicated that polymorphism of CYP7A1 gene led to different response to dietary cholesterol in humans (Wang et al., 1998; Lin et al., 2005).

It was interesting that some clinical studies had also demonstrated that LDL-receptor mutation was related to variation in the serum cholesterol level (Umans-Eckenhausen et al., 2002; Humphries et al., 1991; Haviland et al., 1997). Although no direct evidence found that the mutation on the promoter region in hamsters, the mutation of LDL-receptor gene may disrupt the expression of LDL-receptor or the trafficking of it to the liver cell surface. Less LDL-receptor expression resulted in less cholesterol uptake from blood, which eventually caused the hyper-responsiveness of dietary cholesterol in some individual hamsters. The future investigation should focus on identification of LDL-receptor mutation or DNA polymorphism of CYP7A1 in those hyper-responsive rats and hamsters.

CHAPTER 5

Conclusion

It has been demonstrated that liver plays a very important role in controlling the plasma cholesterol level in response to dietary cholesterol, via two pathways: uptake from blood via LDL-receptor and elimination to bile acids, via CYP7A1. The responsiveness to dietary cholesterol is due to the different ability to utilize these two pathways. The following model explains why rats are hypo-responsive to dietary cholesterol (Figure 5.1). As dietary cholesterol brings into the liver by LDL-receptor, HMG-CoA reductase is inhibited while LXR-α is up-regulated by cholesterol. The LXR-α up-regulates CYP7A1, which converts more cholesterol to bile acids. As biliary cholesterol binds with bile acids in intestine and hindered the enterohepatic circulation of bile acids, a large amount of neutral sterol and bile acids was excreted in feces. Therefore, up-regulation CYP7A1 is due to two mechanisms in rats: (i) stimulation of LXR-α and, (ii) less negative feedback of bile acids. As a result, a cholesterol efflux of cholesterol, driven by conversion of cholesterol to bile acids, occurs in the liver. In response to the net cholesterol efflux, more nSREBP-2 is formed, which it acts as a transcription factor to up-regulate the transcription of LDL-receptor. The up-regulation of both hepatic LDL-receptor and CYP7A1 generates a cholesterol flux from blood to feces. Liver acts as an intermediate to uptake blood cholesterol, which is then being eliminated quickly in feces. Hence, rats are hypo-responsive to dietary cholesterol.

In contrast, hamsters are hyper-responsive to dietary cholesterol because of the following mechanism (Figure 5.2). Dietary cholesterol brings into liver by LDL-receptor, under physiological condition as in rat. In liver, HMG-CoA reductase

is inhibited while LXR-α is not up-regulated by cholesterol. In addition, bile acids are being brought back to liver via enterohepatic circulation and inhibit the expression of CYP7A1. As a result, CYP7A1 is not up-regulated to convert more cholesterol to bile acids and cholesterol flux is not generated in the liver as in rat. Without cholesterol efflux, nSREBP-2 and LDL-receptor are not up-regulated. Hence, hamsters are hyper-responsive to dietary cholesterol because they do not eliminate cholesterol and transport cholesterol from blood to liver efficiently.

Further efforts had made to investigate the mechanism of individual variation of serum cholesterol in response to the same level of dietary cholesterol. The results showed that more CYP7A1 protein expression was correlated with the less serum cholesterol in both rats and hamsters. However, only LDL-receptor protein expression was negatively correlated with serum cholesterol in hamsters. The protein expressions of nSREBP-2, LXR-α and HMG-CoA reductase were not correlated with the serum total cholesterol in both rats and hamsters. High expression of CYP7A1 and LDL-receptor helps individuals eliminate excess cholesterol from body and increase uptake of cholesterol from blood, respectively. Therefore, different response to dietary cholesterol was observed in individuals with various expression level of these proteins, even in the same animal model.

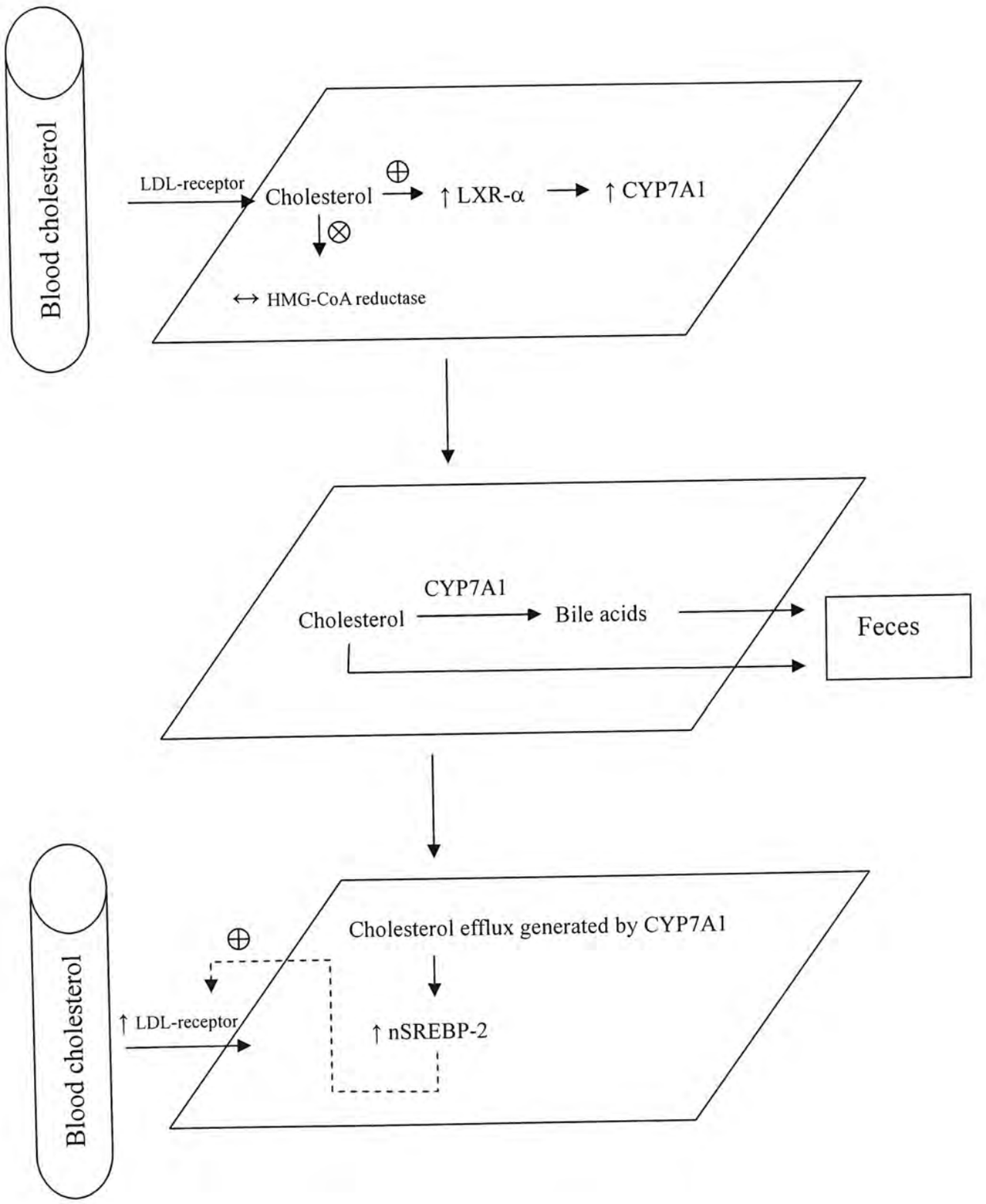

Figure 5.1. The mechanism for the hypo-responsiveness of rat to dietary cholesterol.

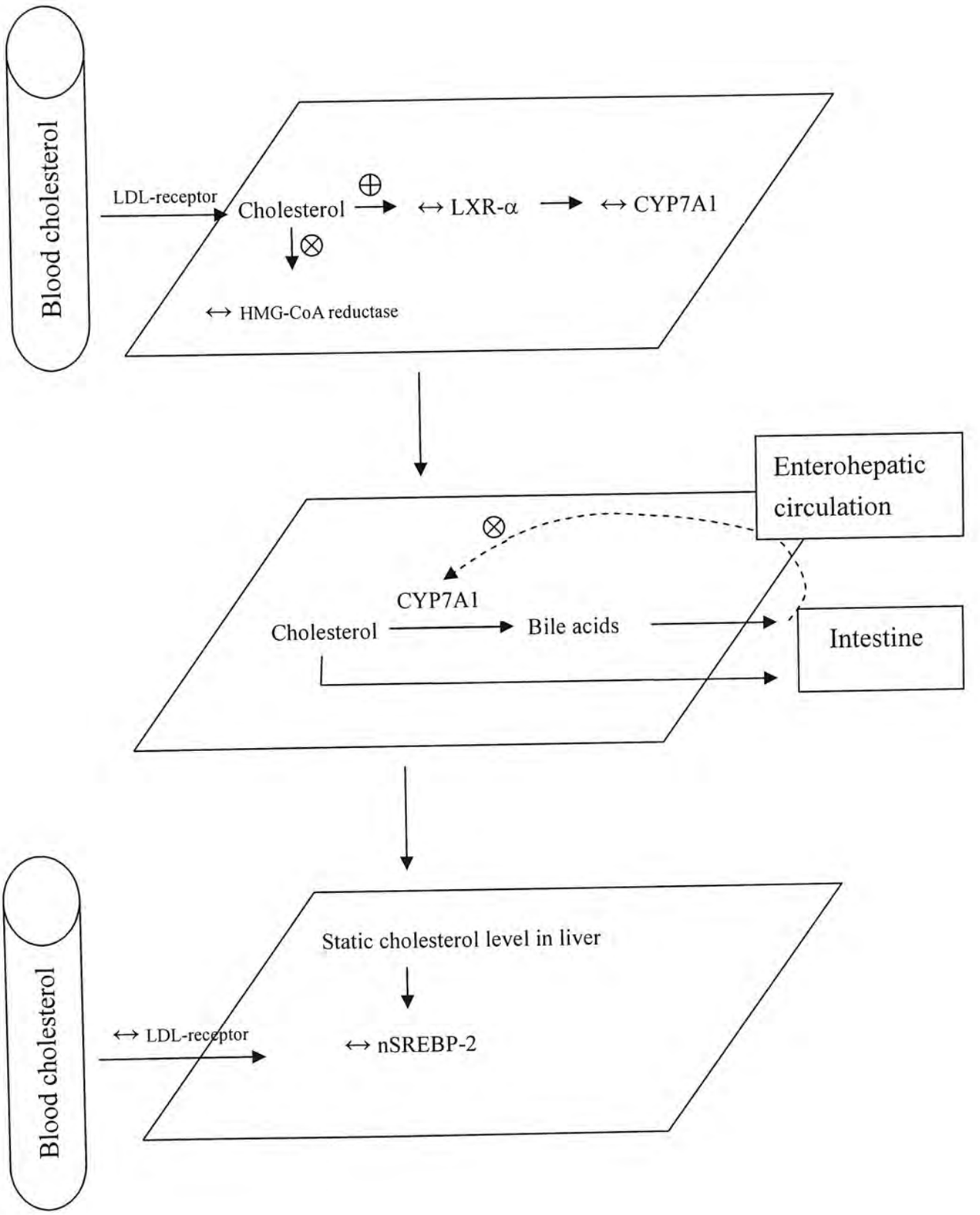

Figure 5.2. The mechanism for the hyper-responsiveness of hamster to dietary cholesterol.

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